REMARKS

Reconsideration and withdrawal of the rejection of all of the claims under 35 U.S.C. 112 for lack of enablement, set forth on pages 2-7 of the Action, as well as the non-statutory obviousness type double patenting rejection over claims 1-6 of US 6,734,176, which issued from parent application 10/320,894, set forth on pages 7 and 8 of the Action, are respectfully requested.

Applicants' claimed invention defines a method for treating a condition that responds to treatment with cannabinoid antagonists selected from the group consisting of schizophrenia, Parkinson's disease, Huntington's chorea, Raynaud's syndrome, alcohol abuse and pain by administering to a patient in need thereof an effective amount of a compound of formula (I) as defined in the claims. These compounds are disclosed in the application to be cannabinoid antagonists and to be effective for treating the recited conditions.

Applicants are submitting herewith copies of the following additional publications that show art-recognition of the efficacy of CB-1 antagonists to treat said conditions:

T. M. Westlake et al., CANNABINOID RECEPTOR BINDING AND MESSENGER RNA EXPRESSIONIN HUMAN BRAIN: AN*IN VITRO* RECEPTOR AUTIRADIOGRAPHY AND *IN SITU* HYBRIDIZATION HISTOCHEMISTRY STUDY OF NORMAL AGED AND ALZHEIMER'S BRAINS, *Neuroscience* Vol 63, No.3, pp. 637-652, 19994 (Exhibit I ... Alzheimer's);

Y. P. Maneuf et al., The Cannabinoid Receptor Agonist WIN 55,212,-2 Reduces D_2 but Not D_1, Dopamine Receptor-Mediated Alleviation of Akinesia in the Reserpine-Treated Rat Model of Parkinson's Disease, EXPERIMENTAL NEUROLOGY 148, 265-270 (1997) (Exhibit II ... Parkinson's); and

Roger G. Pertwee, **Neuropharmacology and therapeutic potential of cannabinoids**, Invited Review, Addiction Biology (2000) 5, 37-46 (Exhibit III ... schizophrenia).

Further, the rejection is not well taken and should be reconsidered and withdrawn. This rejection is couched in terms of enablement – how to make, operability, etc., and the physician as the person of ordinary skill in the art. However, the enablement requirement is more than adequately met in Applicants' specification. Nor is it appropriate to designate the general practitioner physician as the one of ordinary skill in the art to which the present application is addressed. Rather, this application is addressed to scientists in the pharmaceutical industry, the persons who would manufacture the active compounds, develop dosage forms and methods for administering them, and obtain the necessary clearance from the FDA and similar agencies.

Indeed, as the Examiner has stated on page 8 of the Action,

"It would have been obvious to one having ordinary skill in the art at the time of the invention to select any one of the disorders embraced by the genus taught by the reference [U.S. Patent No. 6,734,176], including those instantly claimed, because the skilled chemist would have the reasonable expectation that the compounds to (sic) have the same activity... disorders taught in the reference...."

Could there be a clearer acknowledgement of enablement, given that the disclosure of the reference and that of the present application are substantially identical?

As regards the double patenting rejection set forth on pages 7 and 8 of the Action, Applicants are submitting herewith a terminal disclaimer fully compliant with 37 CFR 3.73 (b), thereby obviating this rejection.

CONCLUSIONS

Favorable reconsideration and prompt Notice of Allowance are earnestly solicited. The examiner is invited to please contact the undersigned with any questions or

comments preferably at the telephone number or e-mail address indicated below.

Respectfully submitted,

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CANNABINOID RECEPTOR BINDING AND MESSENGER RNA EXPRESSION IN HUMAN BRAIN: AN *IN VITRO* RECEPTOR AUTORADIOGRAPHY AND *IN SITU* HYBRIDIZATION HISTOCHEMISTRY STUDY OF NORMAL AGED AND ALZHEIMER'S BRAINS

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Abstract—The distribution and density of cannabinoid receptor binding and messenger RNA expression in aged human brain were examined in several forebrain and basal ganglia structures. *In vitro* binding of [^{3}H]CP-55,940, a synthetic cannabinoid, was examined by autoradiography in fresh frozen brain sections from normal aged humans ($n = 3$), patients who died with Alzheimer's disease ($n = 5$) and patients who died with other forms of cortical pathology ($n = 5$). In the structures examined—hippocampal formation, neocortex, basal ganglia and parts of the brainstem—receptor binding showed a characteristic pattern of high densities in the dentate gyrus molecular layer, globus pallidus and substantia nigra pars reticulata, moderate densities in the hippocampus, neocortex, amygdala and striatum, and low densities in the white matter and brainstem. *In situ* hybridization histochemistry of human cannabinoid receptor, a ribonucleotide probe for the human cannabinoid receptor messenger RNA, showed a pattern of extremely dense transcript levels in subpopulations of cells in the hippocampus and cortex, moderate levels in hippocampal pyramidal neurons and neurons of the striatum, amygdala and hypothalamus, and no signal over dentate gyrus granule cells and most of the cells of the thalamus and upper brainstem, including the substantia nigra. In Alzheimer's brains, compared to normal brains, [^{3}H]CP-55,940 binding was reduced by 37–45% in all of the subfields of the hippocampal formation and by 49% in the caudate. Lesser reductions (20–24%) occurred in the substantia nigra and globus pallidus, internal segment. Other neocortical and basal ganglia structures were not different from control levels. Levels of messenger RNA expression did not differ between Alzheimer's and control brains, but there were regionally discrete statistically significant losses of the intensely expressing cells in the hippocampus. The reductions in binding did not correlate with or localize to areas showing histopathology, estimated either on the basis of overall tissue quality or silver staining of neuritic plaques and neurofibrillary tangles.

Reduced [^{3}H]CP-55,940 binding was associated with increasing age and with other forms of cortical pathology, suggesting that receptor losses are related to the generalized aging and/or disease process and are not selectively associated with the pathology characteristic of Alzheimer's disease, nor with overall decrements in levels of cannabinoid receptor gene expression.

The initial symptoms of a progressive dementia such as Alzheimer's disease (AD) include cognitive changes in orientation, judgment, abstraction and memory.[21] The cognitive changes that occur during the course of AD appear to correlate with the neuronal destruction seen in structures thought to be important in cognitive processing, namely the hippocampus, amygdala, and temporal, parietal and frontal cortices. Presently, neither the degree of atrophy nor the numbers of neuritic plaques and neurofibrillary tangles, the histological hallmarks of AD, have provided an invariant neuropathological correlate of the degree of clinical dementia.[4] Of the many neurotransmitters and biochemical markers that have been shown to be altered in the course of the disease,[20,42,49] as yet no single one has been implicated as being common to all affected neurons.[25]

The cognitive and memory disturbances seen clinically in AD are reminiscent of similar but transient disturbances seen with marijuana usage. The psychoactive constituent of marijuana, Δ^9-tetrahydrocannabinol, produces deficits in attention, concept formation, convergent and divergent thinking, decision-making, and short-term memory.[12,14,30,41] The CNS actions of Δ^9-tetrahydrocannabinol and other active cannabinoid agonists have been attributed to

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Abbreviations: AD, Alzheimer's disease; BSA, bovine serum albumin; GPe, external segment of globus pallidus; GPi, internal segment of globus pallidus; hCNR, human cannabinoid receptor; SSC, standard saline citrate.

their interaction with cannabinoid receptors that are coupled to adenylate cyclase in neuronal plasma membranes.[31] These receptors are present in high concentrations in brain regions that are important for cognition, emotion and memory formation.[28,29] Since these forebrain regions undergo degeneration in AD, we chose to examine the distribution of cannabinoid receptor binding and mRNA in normal and diseased human hippocampus and other selected brain regions as a test of the hypothesis that the cannabinoid system is potentially important in the basic mechanisms of memory processing and in the pathogenesis of the disease.

EXPERIMENTAL PROCEDURES

Tissue collection

Human brain tissues were obtained and processed for routine histopathology through the Department of Pathology, St Louis University School of Medicine. Of the 13 patients examined, seven had previously satisfied DSM-III-R criteria for dementia[3] and were enrolled in the St. Louis University Alzheimer's Disease Brain Bank program (Table 1).

Brains were removed 2–13 h after death and divided in the midsagittal plane; one hemisphere was retained for pathological diagnosis and the other was immediately placed in a −70°C freezer and stored until use. The frozen hemispheres were then blocked so as to obtain entorhinal cortex, hippocampus, basal ganglia, and portions of insular and medial temporal cortex. The blocks were placed in a cryostat and cut in the coronal plane. Sets of serial 15-μm-thick sections were collected and thaw mounted on to gelatin-coated slides, dried briefly on a hot plate at 30°C and stored at −40°C until use. Sets of sections were collected every 1 mm through the rostral half of the hippocampus, the globus pallidus and the striatum at the level of the anterior commissure. Sets of adjacent sections were selected for receptor binding (total and non-specific), for *in situ* hybridization histochemistry (sense and antisense ribonucleotide probes), and for Nissl staining with Cresyl Violet.

Silver stain for degeneration

Another set of adjacent sections was processed for silver staining for neuritic plaques and neurofibrillary tangles.[5] The stain is a modification of the Gallyas technique for demonstrating axonal degeneration using silver impregnation and physical development.[15] The protocol (by R. C. Switzer III) can be obtained in kit form (Chemicon, Temecula, CA). Silver-stained sections were lightly counterstained with Cresyl Violet.

Cannabinoid receptor in vitro *binding*

The *in vitro* binding assay has been described.[28,29] [^{3}H]CP-55,940 (specific activity 79.4 Ci/mmol) was custom radiolabeled by New England Nuclear (Boston, MA). Both CP-55,940 and CP-55,244 (the most potent cannabinoid in the CP series) were obtained from Pfizer, Inc. Unfixed slide-mounted sections were incubated in a solution of 50 mM Tris HCl, pH 7.4, with 5% bovine serum albumin (BSA) and 10 nM [^{3}H]CP-55,940 at 37°C for 2.5 h. Non-specific binding was determined in adjacent sections by addition of 10 μM CP-55,244. Sections were washed twice in a solution of 50 mM Tris HCl, pH 7.4, with 1% BSA for 4 h at 4°C. The sections were then immersed in a 50 mM Tris–HCl, pH 7.4, 0.5% formaldehyde solution at 25°C for 5 min, quickly dipped in deionized water and blown dry. The slides were placed in X-ray cassettes along with tritium standards (high-density ^{3}H Micro-scales, Amersham, Arlington Heights, IL) and exposed to tritium-sensitive film (Hyperfilm-^{3}H, Amersham). All films were developed (D19, Eastman Kodak, Rochester, NY) after 10 days of exposure.

Cannabinoid receptor in situ *hybridization*

A 1.5 kb SstI-XbaI fragment of the human cannabinoid receptor gene (Bonner T. I. unpublished observations) was inserted into the SstI and XbaI sites of the pSP72 vector (Promega Biotech, Madison, WI) to create the plasmid called hCNR p20. The plasmid was linearized with XbaI or EcoRI to make sense or antisense cRNA probes, respectively, corresponding to bases -28 to 1479 of the human cannabinoid receptor cDNA.[17] *In vitro* transcription of the ribonucleotide probes was carried out using SP6 to make the antisense probe and T7 to make the sense probe (Riboprobe System, Promega) in the presence of [α-^{35}S]UTP (sp. act. 1000–1500 Ci/mmol; New England Nuclear). Probes were heat-denatured (90°C, 5 min) prior to addition to hybridization buffer [50% formamide, 4 × SSC (1 × SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.2), 500 μg/ml single-stranded DNA, 250 μg/ml yeast tRNA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA and 10% dextran sulfate].

Sections were prepared for ribonucleotide probe hybridization as described previously.[50,51] Briefly, tissue was fixed, acetylated, dehydrated, defatted and then hybridized overnight at 53°C with 5 × 10^5 c.p.m. of labeled probe per section, treated with RNase A (20 μg/ml, Boehringer Mannheim) for 30 min at 25°C, rinsed, washed sequentially for 60 min in 2 × SSC at 50°C, 60 min in 0.2 × SSC at 55°C, and 60 min in 0.2 × SSC at 60°C, briefly rinsed in a graded

Table 1. Subject information for cases used in this study

Subject	Sex	Age	*Post-mortem* interval (h)	Dementia	Neuropathological diagnosis
AD-B	F	85	2.8	Yes	AD
AD-E	M	85	7.3	Yes	AD
AD-I	M	69	12.3	Yes	AD
AD-J	M	84	13.0	Yes	AD
AD-M	F	84	2.8	Yes	AD
AD-K	M	90	2.5	Yes	AD: multiple infarct dementia; Fahr's disease
AD-A	F	79	6.2	Yes	Progressive subcortical sclerosis
A-L	F	50	11.9	No	Normal
A-G	M	65	2.3	No	Normal
A-F	M	45	9.5	No	Normal
A-C	F	61	8.5	No	Alcoholic
A-H	M	62	5.5	No	Alcoholic
A-D	M	79	7.7	No	Neuritic plaques; Fahr's disease

series of ethanols containing 0.3 M ammonium acetate, and dried.

Slides and standards, both ^{35}S-impregnated brain paste standards of known radioactivity and wet weight and ^{14}C-impregnated plastic standards (American Radiochemicals, St. Louis, MO), were placed in X-ray cassettes and apposed to film (Hyperfilm-βMax, Amersham) for four to seven days. Films were developed (D19) for 5 min at 20°C.

To determine the anatomical localization of probe at the cellular level, sections were dipped in NTB-2 nuclear track emulsion (Kodak) as described previously,[26] exposed for two to six weeks, developed (D19) for 2 min at 16°C and counterstained with Cresyl Violet.

Quantitative film image analysis

Developed films of receptor binding and probe hybridization were illuminated with a light box, and images were digitized using a solid-state video camera and a Macintosh II computer-based system using Image software (Wayne Rasband, Research Services Branch, NIMH). Brain structures appearing on the monitor were identified using the corresponding Nissl-stained section and a human brain atlas,[16] and defined regions were delineated using a mouse cursor control to obtain an average density reading for the outlined structure. A feature called Density Slice was used to eliminate regions containing background labeling from the area to be measured. For the autoradiographs of receptor binding, these were holes or tears in the structure, and for the mRNA autoradiographs they were areas showing no hybridization signal above tissue background. Whenever possible, the densities from three or more sections (total and non-specific) were obtained for each structure and averaged. Based on the known radioactivity of the ^{35}S standards and the ^{14}C standards (these were co-calibrated) relative to their transmittance values, the density measures were converted to nCi/mg tissue wet weight using a best-fit polynomial equation. Using the specific activity of the isotope and the ratio of mg protein/mg tissue (approximately 1:10), these values were then expressed as pmol bound/mg protein. Cell counts of intensely expressing mRNA-positive cells in the hippocampus were made by isolating such cells with the Density Slice operation—the slice boundaries were adjusted so that such cells were selected and moderately expressing cells rejected. All data were expressed as means ± S.D. Statistical significance was determined by two-way analysis of variance (ANOVA) followed by one-way ANOVA for each structure. Post hoc tests used were the Bonferroni–Dunn test for the binding data and Student-Newman-Keuls test for the hybridization data, with significance determined at the 0.05 and 0.01 levels of confidence.

RESULTS

Neuropathological examination (Table 1) revealed that five of seven patients who had died with signs of dementia had abundant senile neuritic plaques and neurofibrillary tangles in the hippocampus and cerebral cortex (frontal and temporal lobes) satisfying quantitative consensus criteria for AD.[33] The sixth patient was diagnosed as having progressive subcortical sclerosis, exhibiting gliosis and astrocytosis of the deep subcortical nuclei. In this brain, the hippocampus was relatively free of pathology; there were some scant neurofibrillary tangles in the hippocampal CA1 subfield and in the entorhinal cortex, otherwise no other plaques or tangles were seen throughout the brain. The seventh brain demonstrated AD pathology but also had multiple lacunar infarcts. Both of these brains were examined in the course of the study, but included separately in the statistical analysis. The duration of dementia for these patients ranged from three to 12 years.

Brain tissues from six non-demented aged patients were also obtained to serve as controls. On neuropathological examination, five of the controls had no demonstrable neuritic plaques or neurofibrillary tangles. The sixth patient had widespread diffuse neocortical plaques and perivascular calcification in the basal ganglia and, therefore, was given the diagnosis of Fahr's disease. In addition, two other patients had been clinically diagnosed as alcoholics. Thus, these last three brains were examined separately in the statistical analysis. As a result of the exclusions, the AD group consisted of five brains, mean age 81 years, whereas the normal group consisted of three brains, mean age 53 years. The five excluded brains had a mean age of 74 years (Table 1).

Receptor binding with [^3H]CP-55,940.

The autoradiographic distribution of [^{3}H]CP-55,940 binding in the human control brains (Fig. 1a–c) was consistent with the previous report describing cannabinoid receptor localization in humans and other species.[29] Total binding was greater than 90% specific. In the brain regions examined, binding densities were greatest in the dentate gyrus molecular layer, the internal segment of the globus pallidus (GPi) and the substantia nigra pars reticulata (Figs 1, 2; Table 2); they were lowest in the thalamus, subthalamus, pons and mesencephalon (Fig. 1).

Quantitative densitometry showed that in various areas of the AD brain, significant decreases in cannabinoid receptor binding occur relative to control (Table 2). The percentage decreases in the hippocampal formation were: entorhinal cortex, 40%; subiculum, 37%; CA1, 38%; dentate hilus, 45%. These decreases were significant at the 0.01 confidence level. The CA3 subfield was the only region of the AD hippocampus not significantly different from control. The substantia nigra pars reticulata and the GPi were significantly decreased in the AD group (24% and 20%, respectively; $P < 0.05$), whereas the putamen and external segment of the globus pallidus (GPe) were not statistically different from control. The caudate exhibited the greatest decrease in receptor binding (49%; $P < 0.01$). The two areas of cerebral cortex examined, an area of insular cortex (Brodmann's areas 13–16) and a medial portion of superior temporal cortex lining the Sylvian fissure at the level of the GPe and GPi (Fig. 1b), showed decreases in cannabinoid receptor binding that were not significant.

The brains that were not included in the statistical analysis had receptor binding densities (Table 2) that tended to correlate positively with the extent of neuropathology. Cell counts were not performed, so pathology cell loss was estimated on the basis of general appearance of the Nissl-stained tissue, overall shrinkage of the structures and the presence

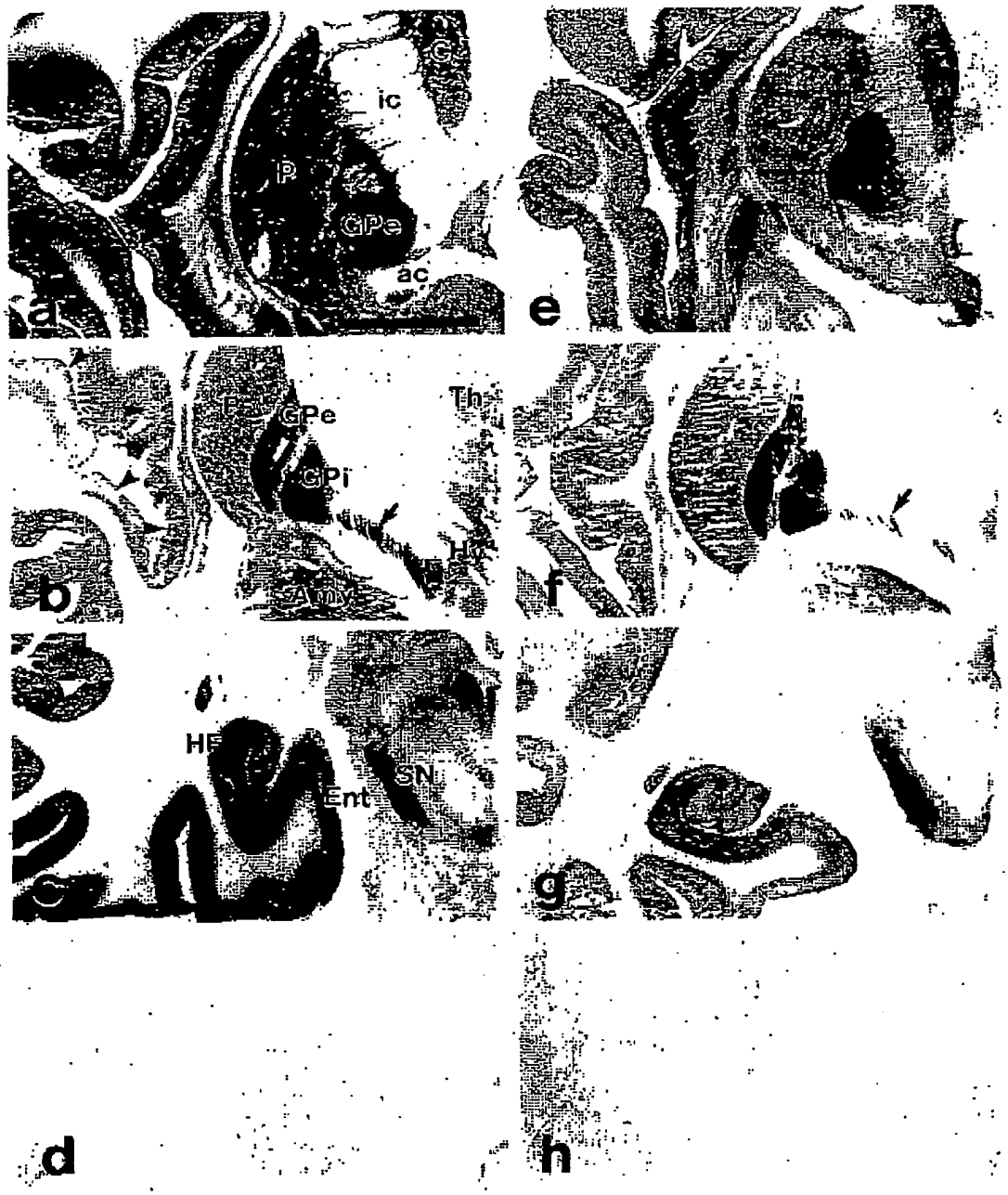

Fig. 1. Autoradiography of 10 nM [^{3}H]CP-55,940 binding in human brain sections cut in the coronal plane. Midline is to the right. On the left (a–d) are film images of a normal brain at three levels: caudate and putamen at the level of the anterior commissure (a), putamen and both segments of the globus pallidus (b), and substantia nigra and rostral hippocampus (c). Non-specific binding in a section adjacent to c is shown in d. On the right are film images of an AD brain at the same levels (e–h correspond to a–d, respectively). Non-specific binding is shown in h. Arrow in b and f points to receptor-dense striatonigral axons. Arrowheads in b point to the insular cortex (medial wall of insula) and temporal cortex (superior, on wall of Sylvian fissure) measured by densitometry. ac, anterior commissure; Amy, Amygdala; C, caudate; Ent, entorhinal cortex; GPe and i, globus pallidus external and internal segments; HF, hippocampal formation; Hy, hypothalamus; ic, internal capsule; P, putamen; SN, substantia nigra; Th, thalamus. Scale bar = 10 mm.

Table 2. Cannabinoid receptor densities in the structures listed, grouped according to diagnosis

Brain region	Control ($n = 3$)	AD ($n = 5$)	EtOH ($n = 2$)	PSS ($n = 1$)	FD ($n = 1$)	AD/MID/FD ($n = 1$)
Entorhinal Cx	1.96 ± 0.15	1.18 ± 0.21**	1.25	1.53	1.35	0.47
Subiculum	2.12 ± 0.28	1.35 ± 0.14**	1.33	2.21	1.47	0.68
CA1	2.29 ± 0.28	1.41 ± 0.16**	1.09	2.28	1.41	0.86
CA3	2.22 ± 0.33	1.67 ± 0.39	1.45	2.42	1.41	0.88
DG ML	2.67 ± 0.14	1.85 ± 0.22**	1.48	2.55	1.57	1.34
DH	1.18 ± 0.06	0.65 ± 0.10**	0.63	1.00	0.57	0.55
SNpr	3.39 ± 0.58	2.59 ± 0.28*	2.40	3.00	2.43	1.64
GPi	3.61 ± 0.50	2.88 ± 0.37*	2.58	2.66	2.81	2.09
GPe	2.52 ± 0.20	2.18 ± 0.67	1.78	1.58	1.60	1.43
Putamen	1.52 ± 0.08	1.20 ± 0.20	1.10	1.24	1.00	0.54
Caudate	1.48 ± 0.36	0.76 ± 0.07**	0.78	0.80	1.05	0.56
Insular Cx	1.57 ± 0.37	1.99 ± 0.10	1.16	1.25	1.29	0.48
Temporal Cx	0.99 ± 0.42	0.73 ± 0.04	0.59	0.55	0.68	0.30

Values are pmol/mg protein and, where applicable, ±S.D. Diagnostic abbreviations: AD, Alzheimer's disease; EtOH, alcoholic; FD, Fahr's disease; MID, multiple infarct dimentia; PSS, progressive subcortical sclerosis. Structure abbreviations: CA1 and 3, hippocampal subfields; Cx, cortex; DG ML, dentate gyrus molecular layer; DH, dentate hilus; GPe and i, globus pallidus external and internal segments; SNpr, substantia nigra pars reticulata. *$P < 0.05$ and **$P < 0.01$; significant difference compared to control.

of degeneration in the silver-stained sections. For example, the brain with progressive subcortical sclerosis had relatively little hippocampal pathology, and thus the hippocampal binding densities were similar to the means of the normal hippocampal values. In other brain regions known to be affected by the disease, such as the GPi, putamen and caudate, this brain had receptor binding densities similar to those means of the AD brains. As for the brains of patients diagnosed as alcoholics, even though these brains had no demonstrable neuritic plaques or neurofibrillary tangles, their receptor binding densities were all strikingly similar to the AD values. Presumably, these brains had some sort of pathology not demonstrably evident using the convential silver stains. The patient who had received no clinical diagnosis of dementia but whose brain had numerous neuritic plaques on neuropathological examination also had receptor binding densities that were similar to values of the AD brains. The patient who had multiple diagnoses, including AD, multiple infarct dementia and Fahr's disease, and had the greatest amount of cell loss had the lowest receptor density values of all structures examined (Table 2).

[^{3}H]CP-55,940 binding was homogeneously distributed throughout neuropil containing plaques and tangles (Figs 2, 3). Within the AD group, there was a very weak correlation between deficits in receptor binding density and the abundance of neuritic plaques and neurofibrillary tangles at the light microscopic level. It would also appear then that the cannabinoid receptor binding deficit is not a reliable correlate of the extent of pathology as defined by plaques or tangles.

In situ hybridization with the human cannabinoid receptor ribonucleotide probe

There was no hybridization of the sense strand—the film images were essentially blank, and the sections were barely visible above slide background and were homogeneous in density (data not shown). The hybridization of the [^{35}S]hCNR antisense probe was highly specific, and the pattern was similar across all the brains examined, though overall differences in signal intensity were large between cases. In all cases, cells showed varying degrees of labeling density in a pattern of distribution across structures that roughly paralleled the distribution of [^{3}H]CP-55,940 binding. Specific details of the normal distribution pattern are shown in Figs 4–8.

The majority of cannabinoid receptor mRNA-expressing neurons were in the cortex, in a pattern that will be described in greater detail below. Subcortical structures examined that contained numerous mRNA-positive cells were the striatum (Fig. 4a–c) and amygdala (Fig. 4b). Overall labeling density was higher in the caudate and putamen than in the nucleus accumbens (Fig. 4a), in contrast to the relatively more homogeneous distribution of [^{3}H]CP-55,940 binding across the major striatal districts (Fig. 4c). The dorsomedial anterior thalamus and the hypothalamus also had significant, though low, levels of hybridization (Fig. 4b).

Microscopic examination of emulsion-coated sections showed details of the cellular labeling that produced the overall patterns. In the caudate (Fig. 5a) and putamen, cells expressing the receptor transcript were pale and medium sized, apparently belonging exclusively to the class of medium-sized striatal projection neuron. The labeling intensity per cell was rather uniform, though not all cells showed hybridization signal. Glial cells, defined by their small size and dark staining, did not have labeling. The pattern in the nucleus accumbens was similar, but the level of expression per cell and the number of expressing cells were lower (Fig. 5b). Probe hybridization in the hypothalamus was restricted to pale-staining neurons of various sizes. Labeled cells were

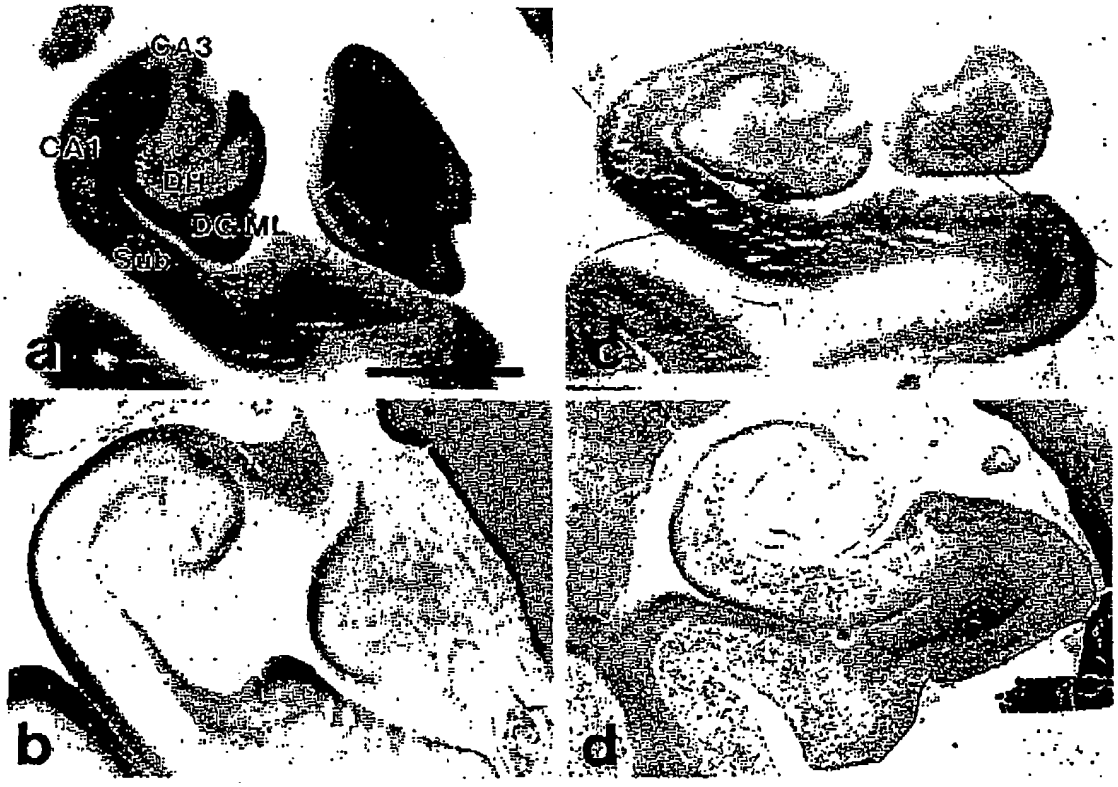

Fig. 2. Autoradiography of [^{3}H]CP-55,940 binding in the hippocampal formation of normal (a) and AD (c) brains. Nearby sections were silver stained to reveal neuritic plaques and neurofibrillary tangles in normal (b) and AD (d) brains. The normal brain is completely devoid of plaques and tangles, with silver deposition occurring only non-specifically in white matter areas. The Nissl counterstain shows the dentate gyrus granule cell layer in both brains. CA1 and 3, fields 1 and 3 of Ammon's horn (Cornu Ammonis); DG ML, dentate gyrus molecular layer; DH, dentate hilus; Sub, subiculum. Scale bar = 5 mm.

homogeneously scattered among non-labeled cells, and each showed similar levels of sparse hybridization (Fig. 5c). An even distribution pattern was seen in the amygdala, but here cells showed great variations in labeling intensity, so that some cells had high levels of mRNA expression (Fig. 5d). These could be seen even in the film images at low magnification (black dots in the amygdala in Fig. 4b). Regions that showed no hybridization signal included the lateral and caudal thalamus, globus pallidus and substantia nigra (Fig. 4b, d and not shown).

Hybridization of the hCNR ribonucleotide probe was found throughout the cerebral cortex. In all areas examined, the most striking feature of the pattern was the presence of intensely expressing mRNA-positive cells scattered amongst a background of cells expressing moderate to low levels of cannabinoid receptor mRNA. The difference in signal level was so large that it could not be quantified. Even at short exposure times (but long enough to measure signal above background in the general population of expressing cells), both the film and the emulsion were saturated, making quantification impossible. It appeared by approximation that these cells showed hybridization intensity at least three orders of magnitude greater than did their neighbors. Such cells were visible as prominent black spots on the film autoradiographs (Figs 4, 7) and could even be seen in emulsion-coated sections viewed with low-magnification bright-field optics (Fig. 6a).

Moderately and intensely mRNA-expressing cells formed laminar patterns of distribution throughout the neocortex. In the areas examined, the supragranular layers concentrated the intensely expressing cells, and infragranular layers contained numerous moderately expressing cells (Figs 6, 7). The appearance of bilaminar labeling was the consequence of relatively greater numbers of intensely expressing cells in layers I and II, a trough of expression (other than scattered intensely labeled cells) in layer III, and a second peak of expressing cells in layers IV, V or VI, depending on the cortical area (Figs 6, 7). Granular insular cortex, for instance, had a prominent second peak in layer IV (Fig. 6a, b), and primary

Fig. 3. Higher magnification views of [^{3}H]CP-55,940 binding (a, c) and silver stains (b, d) in the hippocampal formation of an AD brain. The view in a was magnified in c to show binding in the subiculum and dentate gyrus molecular layer. The corresponding locations and magnifications are shown for the section stained for plaques and tangles (b, d). Relative positions can be determined by aligning blood vessels and patterns. Arrowheads point to corresponding blood vessels in a and c. Scale bars = 2 mm (a, b); 1 mm (c, d).

visual cortex had a weak second peak in layer VI (Fig. 7b).

At high magnification through the microscope, the insular cortical cells expressing cannabinoid receptor mRNAs were varied (Fig. 6). The hybridization signal typically obliterated any information about the radiolabeled cell type underlying it. In layer I, rare cells with intense labeling seemed to be larger than another high-expressing cell type, which was quite small (less than 7 μm diameter). In fact, layer I, which is largely devoid of neurons, was the only site examined where numerous small, sometimes darkly staining cells had moderate or high levels of mRNA expression (Fig. 6c). It was concluded that these must be glial cells. Layer II contained a mixture of cells expressing either moderate or intensely high levels of mRNA (Fig. 6d). Most of these cells appeared to be neurons. Layer III had the lowest level of signal (Fig. 6b). Layer IV had signal over the small, pale cells that correspond to the granule cells of that layer (Fig. 6e). Layer V had moderate and rather homogeneous levels of labeling over the majority of medium-size pale neurons, probably pyramidal cells (Fig. 6f). Intensely labeled cells were very rare. Layer VI looked like layer V in this region of insular cortex, but with less labeling.

The hippocampal formation had similar patterns of hCNR hybridization signal. The intensely labeled cells were largely set apart from the moderately expressing cells, which were confined to the pyramidal cell layers of CA1–CA4 and the subiculum. Thus, intensely labeled cells were scattered throughout the stratum oriens, the dentate hilus, and the molecular layers of both Ammon's horn and the dentate gyrus (Fig. 8a, b, e). The granule cells of the dentate gyrus had no mRNA expression (Fig. 8e). The pyramidal cells of the CA fields and subiculum were almost all labeled, and the labeling intensity, with some exceptions, was moderate (Fig. 8c). The entorhinal cortex showed a laminar distribution of labeling, with elevations in numbers of intensely and moderately expressing cells in both superficial and deep layers (Fig. 8f).

Quantification of the hybridization signal showed great heterogeneity of level across brains. This

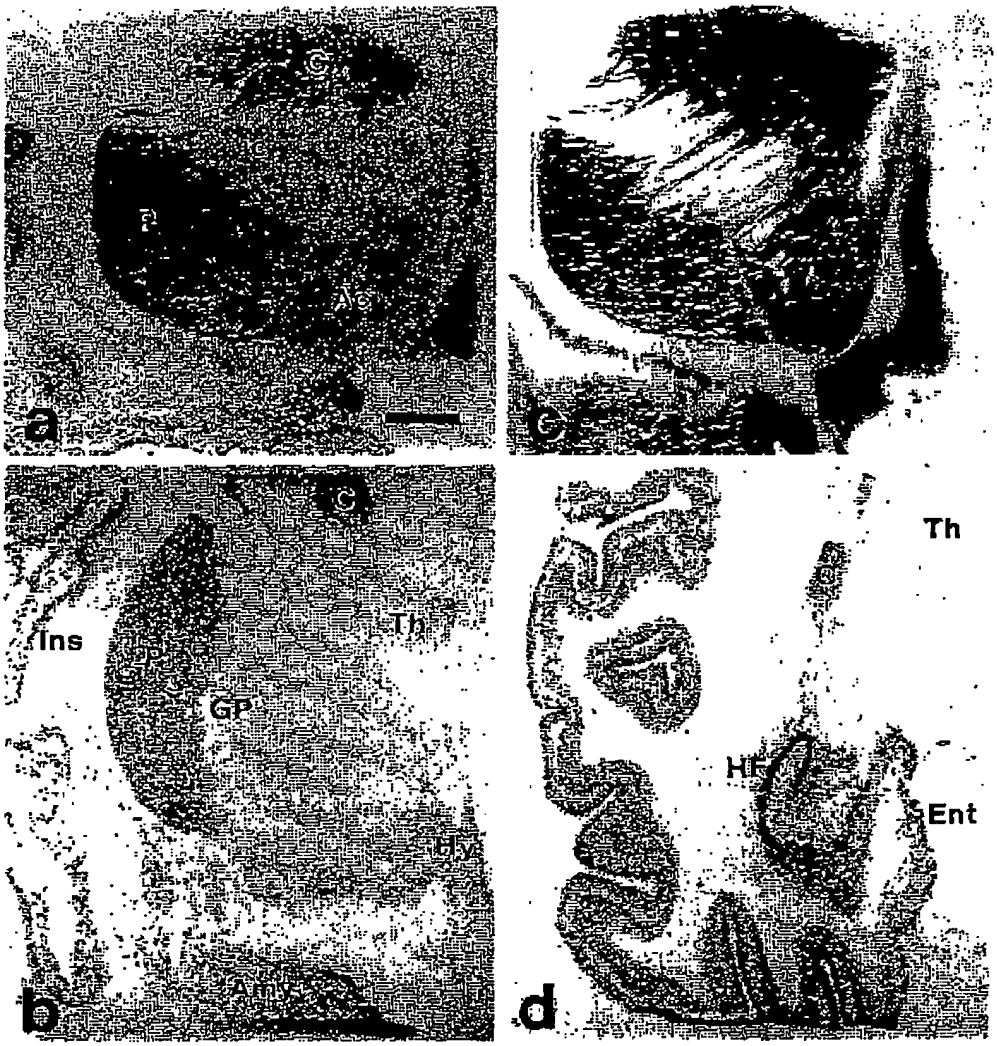

Fig. 4. Cannabinoid receptor mRNA expression, marked by [^{35}S]hCNR *in situ* hybridization (a, b, d) and [^{3}H]CP-55,940 binding (c) in coronal sections of human brain. [^{35}S]hCNR hybridization in the striatum (a) can be compared with the distribution of [^{3}H]CP-55,940 binding in a nearby section (c). Hybridization patterns are also shown at the level of the globus pallidus (b) and hippocampus (d). Amy, amygdala; C, caudate; Ent, entorhinal cortex; GP, globus pallidus; HF, hippocampal formation; Hy, hypothalamus; ic, internal capsule; Ins, insula; P, putamen; Th, thalamus. Scale bar = 5 mm.

heterogeneity did not fall into any grouping according to diagnosis or *post mortem* interval. However, levels of mRNA expression correlated with levels of receptor binding across the 13 brains examined. Within the hippocampal formation (entorhinal cortex, subiculum, fields CA1 and CA3, and the dentate hilus), the correlation between hybridization and binding densities was highly significant ($n = 64$, $R = 0.42$, $P < 0.001$). The number of intensely expressing hCNR mRNA-positive cells was counted and expressed per mm^2 in each area, and the correlation between numbers of high-expressing cells and [^{3}H]CP-55,940 binding densities was even stronger ($n = 64$, $R = 0.57$, $P < 0.0001$) (Fig. 9).

The hCNR mRNA hybridization densities in selected regions of the hippocampal formation, separated according to diagnosis, are given in Table 3. No significant differences were found between normal control and AD brains, though in all areas except the entorhinal area the control values were higher. The largest difference in level of mRNA expression between AD and control brains was found in the caudate, but it was not statistically significant (Table 3). The numbers of intensely expressing hCNR

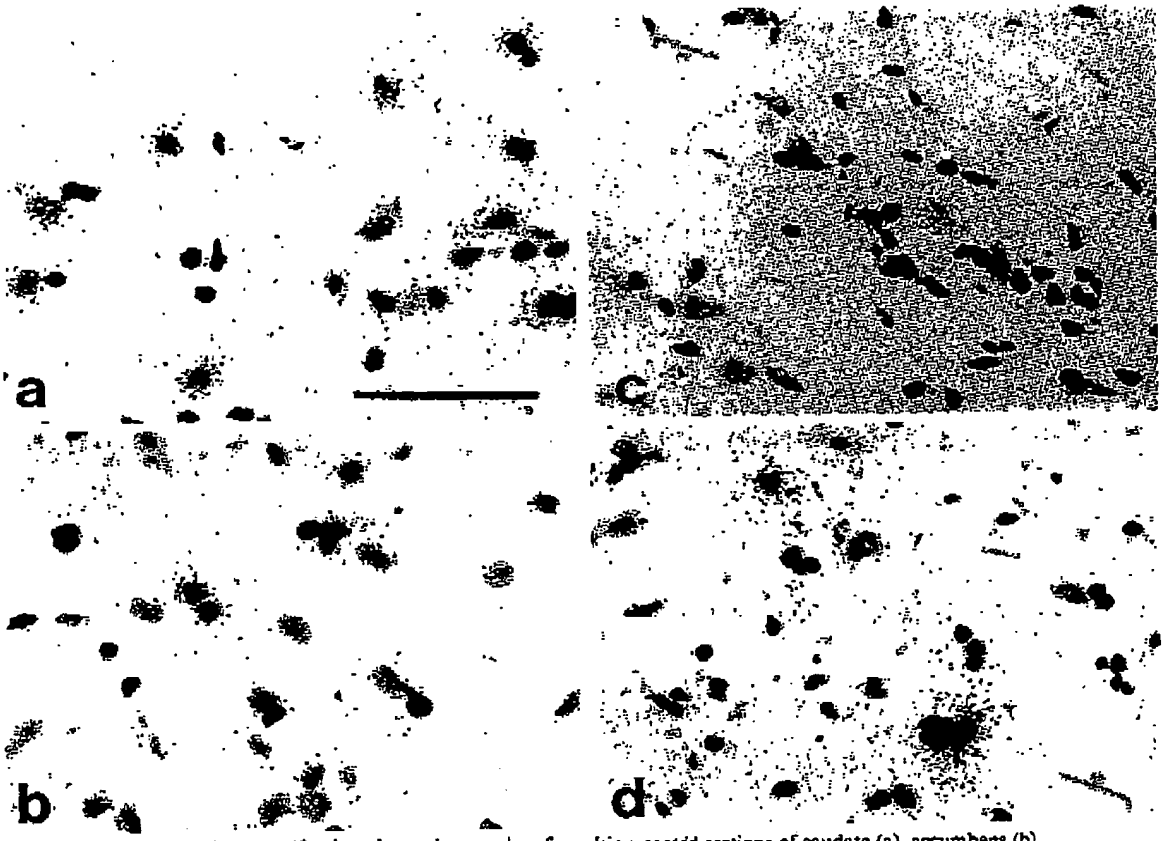

Fig. 5. High-magnification photomicrographs of emulsion-coated sections of caudate (a), accumbens (b), mediobasal hypothalamus (c) and amygdala (d) show [^{35}S]hCNR hybridization signal (clusters of silver grains) overlying pale-staining medium-sized and large neurons. Scale bar = 50 μm.

mRNA-positive cells did show significant differences between control and AD brains in the CA1, CA3 and dentate hilus regions, with the AD cell counts being less than half the control counts (Table 4). An example of the density and pattern of [^{35}S]hCNR labeling in an AD brain is shown in Fig. 8d.

DISCUSSION

Cannabinoid receptor binding

Cannabinoid receptor binding in AD and non-pathological human brains was qualitatively similar, though the AD brains tended to have reduced levels in most of the structures measured. With respect to other cortical regions examined, [^{3}H]CP-55,940 binding throughout the hippocampal formation showed the greatest reduction in AD compared to control brains. Specifically, the entorhinal cortex and dentate hilus had the largest reductions in receptor binding densities. The lack of statistical significance in the CA3 subfield is due to a variability in the densities in the AD group. This variability is consistent with the observation that the CA3 subfield often shows less pathology than the CA1 subfield; the entorhinal cortex, subiculum and CA1 are the areas primarily targeted in AD.[48] This was also apparent in our silver-stained material (Figs 2, 3).

Other quantitative autoradiography studies of receptor distributions in the hippocampus of AD brains have shown a variety of alterations in binding. In the cholinergic system, small reductions in muscarinic binding occur in association with significant cell loss.[23,37,43,44] Similar findings of small reductions in AD hippocampi have been made in the glutamate system for binding to the *N*-methyl-D-aspartate[16,23,25] and quisqualate sites.[33,43] Significant decreases in both types of GABA receptors have been noted in the CA1 and dentate gyrus.[7] In the catecholamine systems, increases in β-adrenoceptors[36] and decreases in D_1 dopamine receptors have been noted.[8] Binding to serotonin$_2$ receptors was found to be unaltered[12] or decreased in the CA1 subfield.[33]

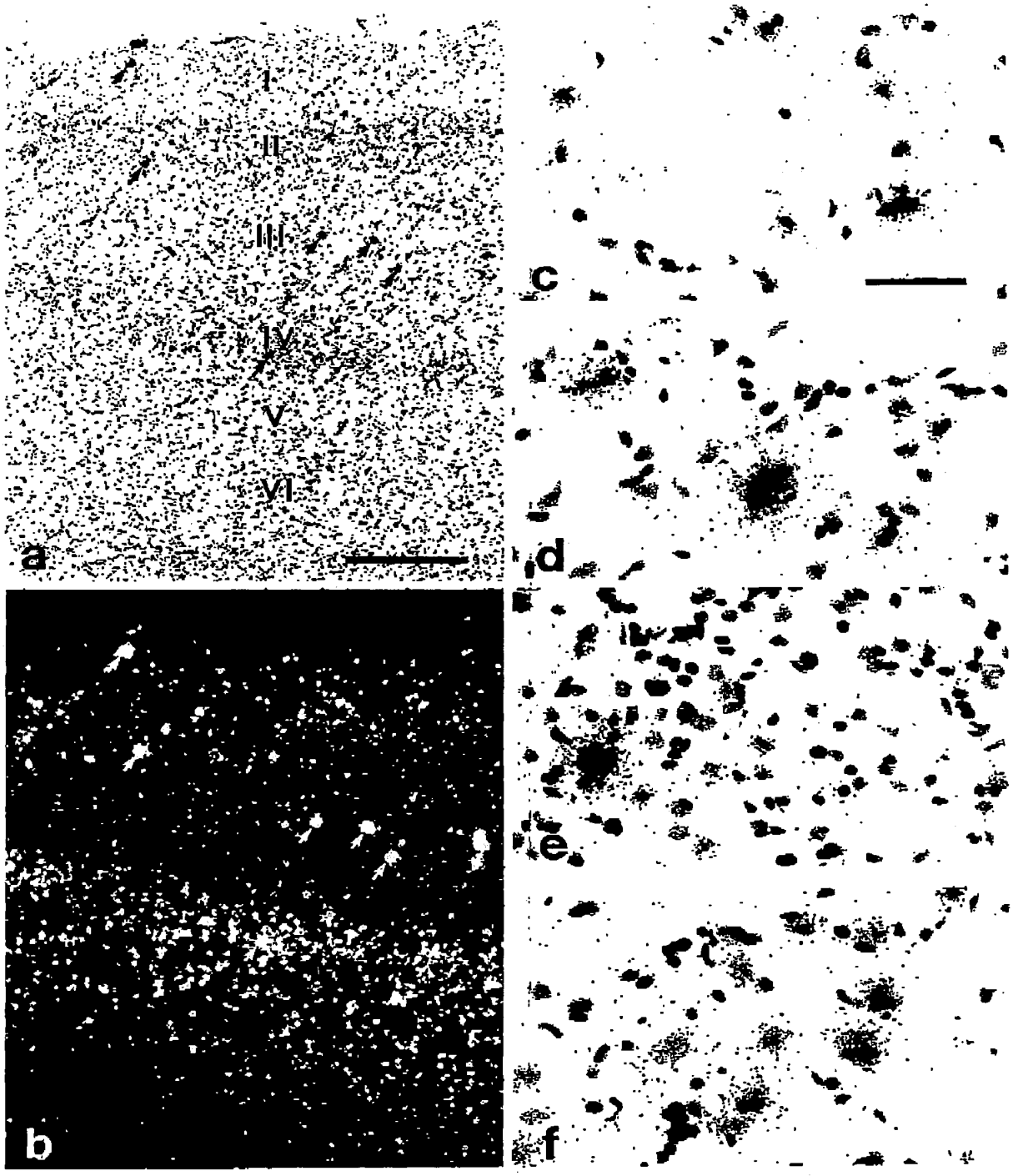

Fig. 6. The pattern of cannabinoid receptor mRNA expression in neocortex is represented in these low- and high-magnification photomicrographs of the granular insular cortex at the level of the globus pallidus. Bright-field (a) and dark-field (b) views of the medial wall of insular cortex show the [^{35}S]hCNR hybridization signal concentrated in the layers shown. The extremely high-expressing cells can be seen even in the bright-field view, and several are marked by arrows. Examples of labeling at high magnification are shown for layers I (c), II (d), IV (e) and V (f). Scale bars = 0.5 mm (a); 50 μm (c).

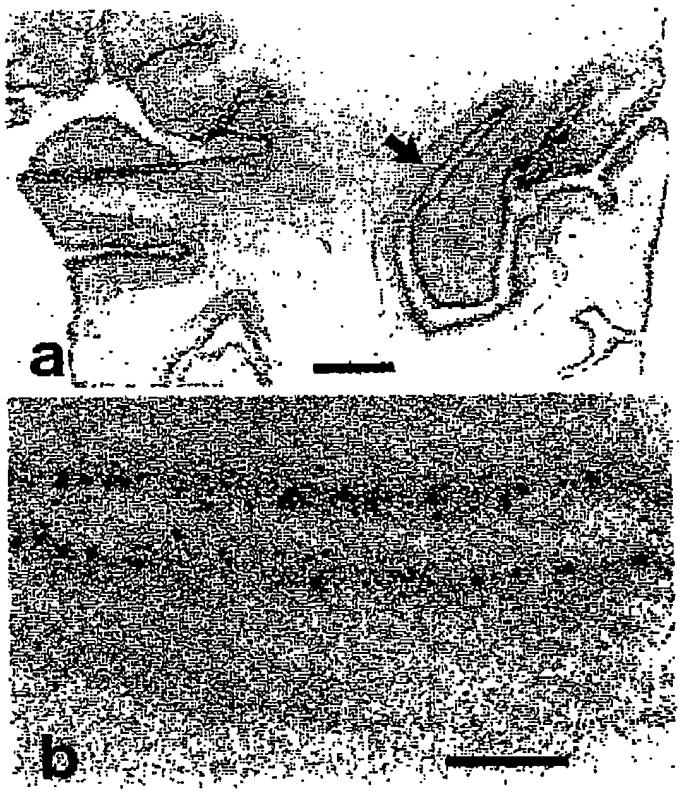

Fig. 7. Cannabinoid receptor mRNA expression in the occipital lobe is seen in a horizontal section (a) that contains the calcarine fissure and primary visual cortex, area 17 (arrow). A higher magnification view of the film image (b) is taken from the point marked by the arrow in a. Scale bars = 5 mm (a); 1 mm (b).

Cannabinoid receptor binding in several basal ganglia structures was selectively reduced in AD brains relative to control (Table 2). A number of neurotransmitter alterations has been reported in the basal ganglia of AD brains.[2,8,9,46] One study found a four-fold greater incidence of extrapyramidal signs and substantia nigra neuropathology in an AD patient population than in a non-AD Parkinson's population of comparable age.[36]

Cannabinoid receptor losses were also noted in the target zones of striatal efferents, i.e. the globus pallidus and substantia nigra. These receptors reside on the axons and terminals of striatal projection neurons.[27] The greater density of cannabinoid receptors in the GPi than in the GPe supports the involvement of cannabinoids in the motor functions of the striatum.[6] The selective losses in the GPi and substantia nigra pars reticulata relative to the GPe suggest another distinction, namely predominance of losses in the D_1 over D_2 dopamine receptor-containing striatal neurons, because D_1 receptors are localized more on nigral/GPi-projecting neurons, whereas D_2 receptors are expressed more on GPe-projecting neurons.[18] In addition, the selectivity suggests greater losses of receptors on GABA/substance P-containing neurons than on GABA/enkephalin-containing neurons.[22,24]

It is also noteworthy that cannabinoid receptor binding reductions were significant in the GPi and not in the GPe (Table 2). In Huntington's disease, the receptor losses are greater in the GPe than in the GPi,[45] suggesting an important pathological difference between Huntington's disease and AD.

Finally, we speculate that the large decrease (49%) in receptor binding in the caudate in AD brains may be related to the profound loss of limbic cortical and hippocampal inputs to this region.[1,47] Whether this reflects loss of receptors on cortical axons or a trans-synaptic effect would be further speculation. It is noteworthy that the largest reduction in cannabinoid receptor mRNA expression level in the AD group compared to control was measured in the caudate (Table 3), though the difference was not significant because of the large variability.

Cannabinoid receptor binding was also reduced in the two alcoholic brains and in the brains showing other forms of neuropathology (Table 2). Cerebral white matter undergoes significant atrophy in the

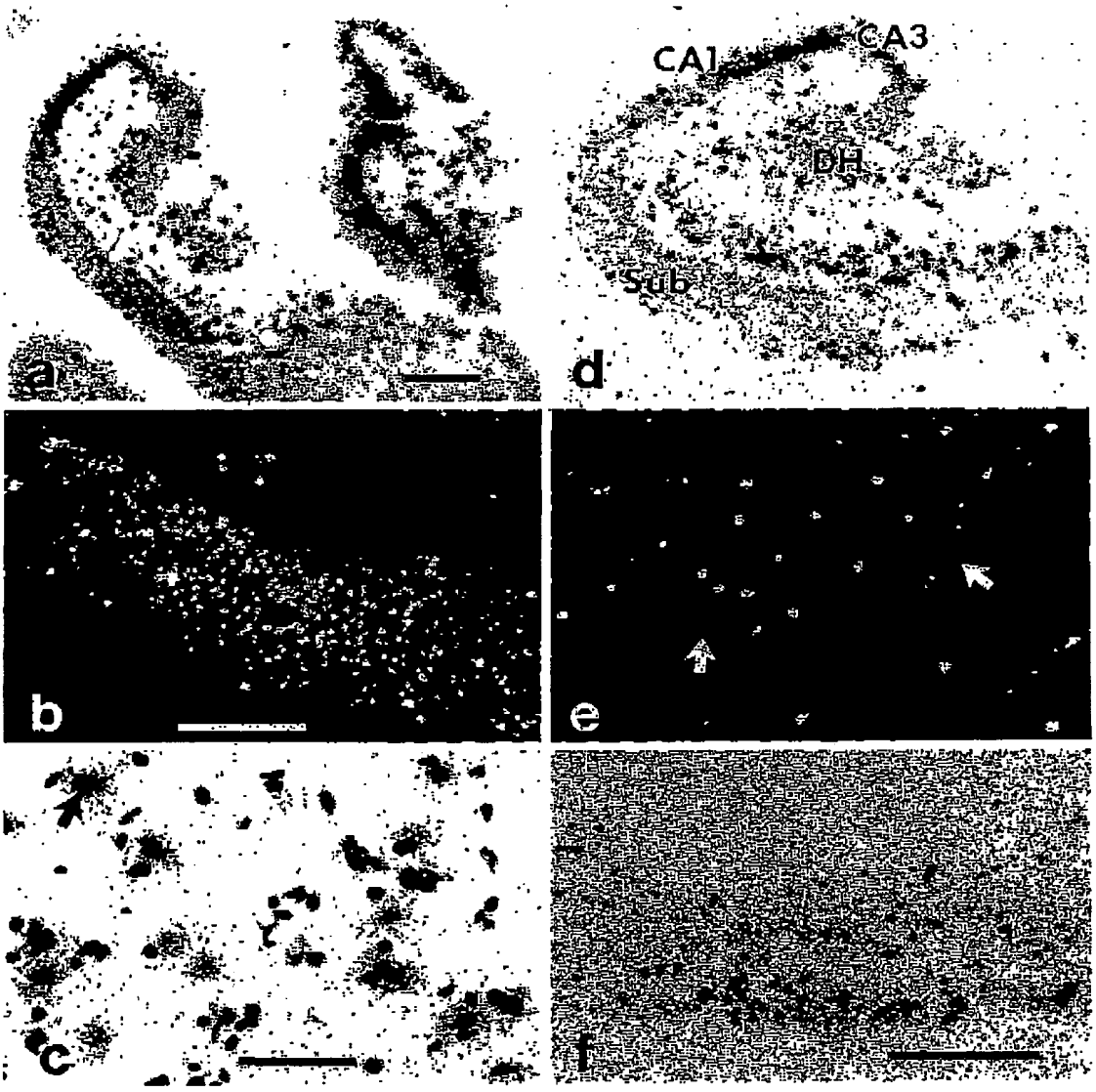

Fig. 8. Cannabinoid receptor mRNA expression in the hippocampal formation of normal (a–c, e, f) and Alzheimer's brain (d) is revealed at several magnifications. The patterns of [^{35}S]hCNR hybridization are described in the text. The level of the pes hippocampi is shown in film images in a and d. CA1 is shown in photomicrographs of emulsion-coated sections in b and c, dentate gyrus is shown in dark-field illumination of emulsion-coated section in e (arrows point to the granule cell layer, which is unlabeled; the dentate hilus is up and the dentate molecular layer is down), and the entorhinal cortex appears in a film image in f (the cortical surface is down). Scale bars = 2 mm (a); 0.5 mm (b); 50 μm (c); 1 mm (f). Magnifications are the same in a and d and in b and e.

brains of both chronic alcoholics and AD patients,[11] suggesting a concomitant loss of cortical neurons. There is an overall reduction in cortical cannabinoid receptor binding in both groups, but the loss in the neocortical areas (insular and medial temporal) is greater in the alcoholic than AD brains (Table 2). There were too few brains in the other categories of pathology to allow speculation, but it is noteworthy that the brain with the most severe pathology had the least amount of cannabinoid receptor binding.

Given the overall losses in [^{3}H]CP-55,940 binding in brains with diverse forms of atrophy, cannabinoid receptor binding may be a fairly consistent and general correlate of neuropathology involving

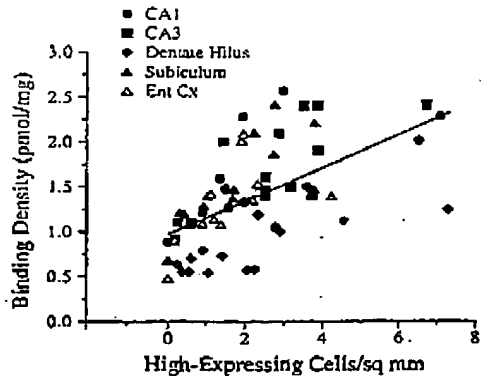

Fig. 9. Correlation of cannabinoid receptor binding densities and numbers of cells expressing high levels of hCNR hybridization is shown for all subjects ($n = 13$) in the five hippocampal regions measured. The regression line is for all the data pooled. The correlation is significant at $P < 0.0001$.

neuronal cell loss. In the AD brains, the areas and layers in which plaques and tangles were selectively localized did not show regionally selective receptor losses in those same districts. At least two other studies have found that receptor binding levels correlate with cell density and not with plaque and tangle density.[32,44] In the present study, the degree of reduction in receptor binding did not closely correlate with the density of plaques and tangles ranked across the five brains. Thus, neither location nor density of plaques and tangles was a correlate of altered cannabinoid binding in AD brains.

Cannabinoid receptor mRNA

Localization of cannabinoid receptor mRNA using the hCNR ribonucleotide probe for the human transcript revealed a pattern of mRNA distribution that is very similar to the pattern seen in the rat.[38,39] The present data greatly extend and clarify *in situ* hybridization data acquired in human brain using an oligonucleotide probe.[37] The similarity of mRNA distribution patterns between rat and human is remarkable, reminiscent of the similarities of receptor binding patterns across the many mammalian species examined, including rat and human.[29] Finally, the pattern similarity and positive quantitative correlation of density levels between cannabinoid receptor mRNA and receptor binding in the human supports the hypothesis that the CNS cannabinoid receptor is a single type and derives from a single gene. Other than the recent discovery of a novel cannabinoid receptor gene that is expressed in immune tissue but not in brain,[42] attempts to clone additional cannabinoid receptor genes by both low-stringency hybridization and polymerase chain reaction techniques have been unsuccessful to date (Lautens L. L., Song Z.-H. and Bonner T. I., unpublished observations).

The great preponderance of cannabinoid receptor mRNA expression is in the forebrain. The cells that express receptor mRNA appear to be neurons in all locations examined except in neocortical layer I, which is almost devoid of neurons. Thus, the numerous small cells in layer I that have moderate and occasionally extremely high levels of mRNA are likely to be glia. The occasional resident neuron in

Table 3. Cannabinoid receptor mRNA densities in the structures listed, grouped according to diagnosis

Brain region	Control ($n = 3$)	AD ($n = 5$)	EtOH ($n = 2$)	PSS ($n = 1$)	FD ($n = 1$)	AD/MID/FD ($n = 1$)
Entorhinal Cx	240 ± 01	290 ± 50	205	270	298	213
Subiculum	400 ± 150	310 ± 80	230	340	318	163
CA1	670 ± 390	400 ± 260	358	399	396	211
CA3	960 ± 540	600 ± 600	421	555	677	265
DH	400 ± 90	300 ± 160	196	295	343	175
Putamen	370 ± 120	270 ± 160	254	513	207	50
Caudate	420 ± 220	200 ± 130	220	202	191	—
Insular Cx	310 ± 60	280 ± 50	225	400	312	99
Temporal Cx	330 ± 70	240 ± 60	205	215	182	59

Values are d.p.m./mg and, where applicable, ±S.D. No differences were significant. Abbreviations as in Table 2.

Table 4. Counts of cells expressing extremely high levels of cannabinoid receptor mRNA in the structures listed, grouped according to diagnosis

Brain region	Control ($n = 3$)	AD ($n = 5$)	EtOH ($n = 2$)	PSS ($n = 1$)	FD ($n = 1$)	AD/MID/FD ($n = 1$)
Entorhinal Cx	1.9 ± 0.1	1.6 ± 1.5	1.02	2.32	2.21	0
Subiculum	2.6 ± 0.3	1.4 ± 1.0	1.32	3.59	3.78	0
CA1	5.5 ± 2.2	2.0 ± 1.1**	2.15	1.95	1.98	0
CA3	4.8 ± 1.6	2.1 ± 1.1*	1.39	3.51	3.74	0.18
DH	4.7 ± 2.5	1.2 ± 0.7*	0.50	2.89	2.07	0.56

Values are mean counts per sq mm ± S.D. (where applicable). *$P < 0.05$ and **$P < 0.01$: significant difference compared to control. Abbreviations as in Table 2.

layer I, the horizontal cell of Cajal, is a likely candidate for the occasional medium-sized neuron that has high mRNA levels. Elsewhere in the neocortex, the cells expressing cannabinoid receptor mRNA appear to be pale staining and medium sized, suggesting that they are neurons, both of the pyramidal and non-pyramidal types. Cannabinoid receptor mRNA expression in the subcortical structures examined appears to be exclusively neuronal, as determined from high magnification analysis (Fig. 5). These cells transcribe mRNA for receptor synthesis and deposition on local processes and axons in the well-described projection zones. Thus, as has been documented for the rat[27] and human,[19,45] pallidal and nigral receptor binding resides on the axons of striatal projection neurons. Likewise, the sparse binding seen in the thalamus (Fig. 1b, c) may reside on axons of mRNA-positive cells in layer VI of the neocortex, because most areas of the thalamus do not appear to express cannabinoid receptor mRNA (Fig. 4b, d). Hypothalamic and amygdaloid binding, on the other hand, can be best attributed to receptor synthesis in resident neurons, which are shown to have low to moderate levels of mRNA expression (Fig. 5b, d).

The locations and densities of cannabinoid receptor binding correlate well with those of mRNA expression in the neocortex. Both binding and expression are fairly homogeneously distributed across cortical areas, and peaks of both are typically observed in superficial and deep layers. In the striatum, the disparity of mRNA levels between the caudate and putamen on the one hand and the nucleus accumbens on the other does not have a clear correlate in binding, which is rather homogeneous across the striatal districts (Fig. 4a, b). However, the mRNA gradient does comport with the binding gradient seen in the rat,[28] suggesting that the apparent species difference in binding is not so pronounced.

Only the cortex, and to lesser extent the amygdala, contains scattered cells with the characteristic extremely high expression levels, which are estimated to be at least three orders of magnitude greater than the neighboring mRNA-positive cells. It is impossible to determine what component and percentage of the receptor distribution pattern is derived from protein translated from these abundant transcripts. One clue comes from the hippocampus, where the dentate gyrus molecular layer has a high level of receptor binding that does not reside on granule cell dendrites in that layer because there is no mRNA expression in granule cells. Likely sources of the binding are the scattered extremely high-expressing cells residing in the molecular layer and dentate hilus, which would send processes, both dendrites and axons, diffusely into the molecular layer. Other candidates for sources of the binding are axons of extrinsic neurons, but these have laminar domains of termination rendering them unlikely sources. In the present study, the declines in receptor binding in the dentate hilus and molecular layer (Table 2) correlate with disappearance of the high-expressing cells (Table 4), supporting the possibility that these cells may be the major source of the binding throughout the dentate gyrus.

Densitometric analysis of cannabinoid receptor mRNA levels in specific brain regions showed marked variations in levels between brains. Consequently, none of the differences between the average densities of AD and control brains was significant (Table 3). The lack of difference for the structures examined suggests that receptor binding losses on axons and terminals of striatal and hippocampal neurons occur without changes in mRNA expression levels in the parent cell bodies and without significant losses of the moderately expressing cells themselves.

SUMMARY AND CONCLUSIONS

In several hippocampal and basal ganglia structures, cannabinoid receptor densities were reduced in AD patients compared to aged normal brains. These reductions do not appear to be associated with the neuronal plaques and tangles which characterize AD. Therefore, decreased receptor densities may be due to other degenerative mechanisms. The decreased binding may reflect neuronal cell loss or receptor down-regulation. This latter explanation is difficult to evaluate because the overall levels of receptor mRNA varied greatly between brains. However, the reductions in cannabinoid receptors in the dentate gyrus may be due to the loss of interneurons that express extremely high levels of cannabinoid receptor mRNA.

It is possible that the mechanisms responsible for producing acute cognitive and memory deficits experienced by users of marijuana involve hippocampal neurons that degenerate in AD. This speculation paradoxically suggests that a decrease in cannabinoid receptor densities may exacerbate the cognitive and memory deficits that plague AD patients. The nature of the consequences of reduced cannabinoid receptor densities and the possibility of compensatory increases in levels of endogenous cannabinoids in AD patient brains should be explored before speculating further on the significance of these findings.

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INVITED REVIEW

Neuropharmacology and therapeutic potential of cannabinoids

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Abstract

Mammalian tissues contain at least two types of cannabinoid receptor, CB_1, found mainly on neurones and CB_2, found mainly in immune cells. Endogenous ligands for these receptors have also been identified. These "endocannabinoids" and their receptors constitute the "endogenous cannabinoid system". Two cannabinoid receptor agonists, Δ^9-tetrahydrocannabinol and nabilone, are used clinically as anti-emetics or to boost appetite. Additional therapeutic uses of cannabinoids may include the suppression of some multiple sclerosis and spinal injury symptoms, the management of pain, bronchial asthma and glaucoma, and the prevention of neurotoxicity. There are also potential clinical applications for CB_1 receptor antagonists, in the management of acute schizophrenia and cognitive/memory dysfunctions and as appetite suppressants. Future research is likely to be directed at characterizing the endogenous cannabinoid system more completely, at obtaining more conclusive clinical data about cannabinoids with regard to both beneficial and adverse effects, at developing improved cannabinoid formulations and modes of administration for use in the clinic and at devising clinical strategies for separating out the sought-after effects of CB_1 receptor agonists from their psychotropic and other unwanted effects.

The plant *Cannabis sativa* is the source of a set of more than 60 oxygen-containing aromatic hydrocarbon compounds called cannabinoids. One of these, Δ^9-tetrahydrocannabinol (Δ^9-THC), is responsible for most of the psychotropic properties of cannabis.[1] It is also of interest because it is one of just two cannabinoids to be licensed for medical use. Thus Δ^9-THC, as the oral preparation dronabinol (Marinol), is available in the United States for the suppression of nausea and vomiting provoked by anticancer drugs and for the reversal, through appetite stimulation, of body weight loss experienced by AIDS patients. The other cannabinoid that it is permissible to use clinically is nabilone (Cesamet), a synthetic analogue of Δ^9-THC that is also given by mouth. This compound is licensed for use in the United Kingdom, again to suppress nausea and vomiting produced by cancer chemotherapy. Because psychotropic cannabinoids have high lipid solubility and low water solubility they were long thought to owe their pharmacological properties to an ability to perturb the phospholipid constituents of biological membranes.[1] However, all this changed

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with the discovery, in the late 1980s, of specific cannabinoid receptors.

There are at least two types of cannabinoid receptor: CB_1 and CB_2.[2,3] Both are coupled through G_i or G_o proteins, negatively to adenylate cyclase and positively to mitogen-activated protein kinase. In addition, CB_1 receptors are thought to be coupled negatively to N- and P/Q-type calcium channels and positively to A-type and inwardly rectifying potassium channels, again through G_i or G_o proteins.[2] Under certain conditions, CB_1 receptors may also act through G_s proteins to activate adenylate cyclase.[4] More speculatively, CB_1 receptors may mobilize arachidonic acid and close 5-HT_3 receptor ion channels.[2] Cannabinoids can also close sodium channels but the question of whether this effect is receptor-mediated has still to be addressed.[2] CB_1 and CB_2 receptors have both been cloned, the predicted amino acid sequences of these two receptor types showing a similarity of about 44% (35% to 82% within the individual transmembrane domains).[3] CB_1 and CB_2 receptors also differ in their distribution pattern. CB_1 receptors are found mainly on neurones in the brain, spinal cord and peripheral nervous system[2] where they inhibit neurotransmitter release when activated. Thus results from *in vivo* experiments indicate that CB_1 receptors mediate inhibition of acetylcholine release in rat medial-prefrontal cerebral cortex and hippocampus.[6,7] There is also evidence for CB_1 receptor-mediated inhibition of the release of (a) acetylcholine from rat hippocampal slices, guinea-pig small intestine and mouse bladder, (b) glutamate from rat hippocampal cultured neurones, (c) γ-aminobutyric acid from slices of rat substantia nigra and striatum, (d) ATP from mouse vas deferens, (e) dopamine from guinea-pig retinal discs and rat striatal slices and (f) noradrenaline from guinea-pig retinal discs, from slices of guinea-pig and human hippocampus, from guinea-pig cerebral cortical, cerebellar and hypothalamic slices and from rat heart, mouse urinary bladder and mouse vas deferens.[2,3,8–14] The distribution of CB_1 receptors within the CNS is consistent with the ability of psychotropic cannabinoids to impair cognition and memory and to alter the control of motor function and the perception of pain. Thus the cerebral cortex, hippocampus, caudate-putamen, substantia nigra pars reticulata, globus pallidus, entopeduncular nucleus, cerebellum, central grey substance and dorsal horn of the spinal cord all contain significant numbers of CB_1 receptors.[2,3] In contrast, CB_2 receptors occur mainly in immune cells where they may mediate an immunosuppressant effect.[2,3]

The realization that psychotropic cannabinoids act through receptors was followed by the demonstration, in 1992, that mammalian tissues can also produce agonists for these receptors. The most important of these "endocannabinoids" are arachidonoylethanolamide (anandamide) and 2-arachidonoyl glycerol.[2,3] Consistent with the hypothesis that both these compounds serve as neuromodulators or neurotransmitters is evidence that they are synthesized within neurones, that they can undergo depolarization-dependent release from neurones and that once released they are rapidly removed from the extracellular space.[3,15–17] For anandamide, such removal seems to depend on a carrier-mediated, saturable uptake process that is followed, within the cell, by hydrolysis to arachidonic acid and ethanolamine.[15,18,19] This hydrolysis is catalysed by a microsomal enzyme, fatty acid amide hydrolase,[3,19–22] which also catalyses the hydrolysis of 2-arachidonoyl glycerol.[17,19,23] The presence of an uptake process for 2-arachidonoyl glycerol in neurones remains to be established. These findings have sparked interest in the development of drugs that selectively inhibit the tissue uptake or metabolism of endocannabinoids. One promising compound is *N*-(4-hydroxyphenyl)arachidonylamide (AM404). This has been reported to inhibit anandamide accumulation in rat neurones and astrocytes *in vitro* without also activating CB_1 receptors or inhibiting anandamide hydrolysis, and to enhance cannabimimetic effects of anandamide both *in vitro* and *in vivo*.[24] Cannabinoid CB_1 and CB_2 receptors and endocannabinoids together constitute the "endogenous cannabinoid system".

The discovery of cannabinoid receptors has prompted the development of selective CB_1 and CB_2 receptor agonists and antagonists[2,3,25] (see also references in Table 1). Selective CB_1 receptor antagonists include SR141716A and the less potent LY320135.[2,3,25,36] The most important CB_2 receptor antagonist to have been developed is SR144528.[37]. This is highly potent, its affinity for CB_2 receptors matching that of SR141716A for CB_1 receptors. When administered by itself, SR141716A produces effects in some biological systems that are opposite in direction to those

produced by cannabinoid receptor agonists.[2] These, presumably, are biological systems in which the endogenous cannabinoid system is tonically active. Such activity may reflect ongoing release of endocannabinoids and/or the presence of constitutively active cannabinoid receptors, there being evidence that SR141716A is not a pure antagonist but rather an inverse agonist.[38,39] The CB_2-selective ligand, SR144528, may also be an inverse agonist.[37] Agonists with the greatest selectivity for CB_1 receptors are O-689, O-585 and methanandamide[3,34] (Table 1) while important CB_2-selective agonists include L759633 and L759656[2,25,33] (Table 1). Of the three cannabinoid receptor agonists that are most commonly used as pharmacological tools, (+)–WIN55212, shows some degree of CB_2 selectivity whereas CP55940 and Δ^9-THC bind equally well to CB_1 and CB_2 receptors (Table 1). Of these, Δ^9-THC has the least efficacy, particularly at CB_2 receptors.[3,35,40,41] Anandamide also appears to have less efficacy at CB_2 than at CB_1 receptors.[2,3] On the other hand, the dimethylheptyl derivative of D^8-THC-11-oic acid (CT3) seems to have greater efficacy at CB_2 than at CB_1 receptors, as measured by cannabinoid receptor-mediated inhibition of adenylate cyclase in cultured cells,[35] even though it has greater affinity for CB_1 than CB_2 receptors (Table 1). It remains to be established whether CT3 acts on CB_2 receptors to produce its anti-inflammatory and antinociceptive effects.[35,42]

One endogenous fatty acid amide whose pharmacological status as a cannabinoid receptor ligand remains to be established is palmitoylethanolamide. Showalter *et al.*[34] have found that this compound has little affinity for cloned human CB_2 receptors and it has also been reported not to bind to CB_1 receptors.[43] On the other hand, Facci *et al.*[44] have reported that palmitoylethanolamide inhibits 5-HT release from a cell line known to express CB_2 receptors (RBL-2H3 cells), and that it readily displaces [^{3}H](+)–WIN55212 from specific binding sites on membranes from these cells. More recently, Calignano *et al.*[45] found that when palmitoylethanolamide was injected into the hind paws of mice, it suppressed behavioural signs of hyperalgesia induced by intraplantar injection of dilute formalin and that this antinociceptive effect of palmitoylethanolamide could be readily blocked by the CB_2 antagonist, SR144528, but not by the CB_1 antagonist, SR141716A. Taken together, these findings suggest that palmitoylethanolamide may serve as an agonist for a "CB_2-like" receptor.

The discovery of the endogenous cannabinoid system has been paralleled by a growing interest in the possibility that cannabinoid receptor ligands may have therapeutic applications in addition to those of anti-emesis and appetite stimulation (see above). In particular, CB_1 receptor antagonists could prove to be of value as appetite suppressants, in the management of acute schizophrenia and/or for ameliorating cognitive/memory dysfunctions associated with disorders such as Alzheimer's disease.[25,46–48] As detailed elsewhere, there is also evidence that CB_1 receptor agonists have potential for the management of glaucoma, bronchial asthma and pain and for the suppression of muscle spasticity/spasm associated with conditions such as multiple sclerosis or spinal cord injury.[49,50] For several of the potential clinical applications of CB_1 receptor agonists, including their possible use against muscle spasticity/spasm and chronic pain, the evidence is already sufficient to warrant the setting-up of clinical trials that will test cannabinoid efficacy both objectively and conclusively.[49,50] An answer to the question of whether drugs that activate or block CB_2 receptors have therapeutic potential, for example as anti-inflammatory agents or immunomodulators, must await a more complete characterization of this component of the endogenous cannabinoid system.

Not all effects of cannabinoids are mediated by receptors and some of these may also have clinical applications. Of particular importance is evidence from whole animal and tissue experiments that the psychotropically inactive cannabinoid, (+)–11-hydroxy-Δ^8-THC-dimethyl-heptyl (HU-211, dexanabinol) is effective against neuropathic pain,[51] optic nerve neuropathies,[52] tumour necrosis factor-α-mediated septic shock[53] and neurotoxicity induced by glutamate, NMDA or quisqualate[54,55] and that it protects from neurotoxic changes in the brain arising from ischaemia,[56,57] closed head injury[58,59] or pneumococcal meningitis.[60] These neuroprotective effects are not cannabinoid receptor-mediated as HU-211 is not a ligand for these receptors. Instead, they may depend on an ability of HU-211 to act as a non-competitive NMDA receptor antagonist,[54,55,61,62] as a scavenger of oxygen-derived free radicals[59] and/or as an inhibitor of depolarization-evoked calcium fluxes[59] or of tumour necrosis factor-α production.[53]

Table 1. *The abilities of certain ligands to displace [^{3}H]CP55940 or [^{3}H]HU-243* from human (h), rat (r) or mouse CB_1 and CB_2 receptors*

Displacing ligand	CB_1 membranes	CB_2 membranes	CB_1 K_i (nM)	CB_2 K_i (nM)	Affinity ratio CB_1/CB_2	Reference
SR141716A	hCB_1 in L cells	hCB_2 in CHO cells	11.8	13200	1119	36
	hCB_1 in L cells	hCB_2 in AtT-20 cells	11.8	973	83	27
	hCB_1 in CHO cells	hCB_2 in CHO cells	12.3	702	57	34
LY320135	hCB_1 in L cells	hCB_2 in CHO cells	141	14900	106	36
Methanandamide	Rat forebrain	Mouse spleen	20	815	41	31
O-585	hCB_1 in CHO cells	hCB_2 in CHO cells	8.6	324	38	34
O-689	hCB_1 in CHO cells	hCB_2 in CHO cells	5.7	132	23	34
CT3	rCB_1 in COS cells*	hCB_2 in COS cells*	32.3	170.5	5.3	35
Anandamide	hCB_1 in CHO cells	hCB_2 in CHO cells	89	371	4.2	34
	hCB_1 in L cells	hCB_2 in AtT-20 cells	543	1940	3.6	27
	rCB_1 in COS cells*	hCB_2 in COS cells*	252	581	2.3	26
2-Arachidonoyl glycerol	rCB_1 in COS cells*	hCB_2 in COS cells*	472	1400	3.0	26
	CB_1 in COS cells*	CB_2 in COS cells*	58.3	145	2.5	32
Δ^9-THC	hCB_1 in L cells	hCB_2 in AtT-20 cells	53.3	75.3	1.4	36
	rCB_1 in COS cells*	hCB_2 in COS cells*	39.5	40	1.0	29
	hCB_1 in CHO cells	hCB_2 in CHO cells	40.7	36.4	0.9	34
	rCB_1 in COS cells*	hCB_2 in COS cells*	80.3	32.2	0.4	35
Nabilone	hCB_1	hCB_2	1.84	2.19	1.2	28
CP55940	hCB_1 in CHO cells	hCB_2 in CHO cells	5	1.8	0.36	33
	hCB_1 in L cells	hCB_2 in AtT-20 cells	3.72	2.55	0.69	27
	hCB_1 in CHO cells	hCB_2 in CHO cells	0.58	0.69	1.2	34
(+)-WIN55212	hCB_1 in CHO cells	hCB_2 in CHO cells	1.89	0.28	0.15	34
	hCB_1 in L cells	hCB_2 in AtT-20 cells	62.3	3.3	0.053	27
	hCB_1	hCB_2	123	4.1	0.033	30
L759633	hCB_1 in CHO cells	hCB_2 in CHO cells	1043	6.4	0.0061	33
	hCB_1	hCB_2	15850	20	0.0013	28
L759656	hCB_1 in CHO cells	hCB_2 in CHO cells	4888	11.8	0.0024	33
	hCB_1	hCB_2	>20000	19.4	<0.00097	28
SR144528	hCB_1 in CHO cells	hCB_2 in CHO cells	437	0.60	0.0014	37
	hCB_1 in CHO cells	hCB_2 in CHO cells	>10000	5.6	<0.00056	33

In view of the neuroprotective properties of HU-211, it is noteworthy that Hampson *et al.*[63] have reported that Δ^9-THC and the non-psychotropic plant cannabinoid, cannabidiol, are also neuroprotective, as measured by prevention of neurotoxicity induced in cortical neurones by reactive oxygen species or by glutamate (through NMDA/AMPA/kainate receptors). The effects of Δ^9-THC and cannabidiol on glutamate-induced neurotoxicity observed in these experiments did not appear to be CB_1 receptor-mediated as they were not prevented by the CB_1 receptor antagonist, SR141716A. In an earlier investigation, Skaper *et al.*[64] reported that the cannabinoid receptor agonists, Δ^8-THC, (+)-WIN55212 and nabilone, protect against glutamate-induced neurotoxicity in cerebellar granule neurones.[64] However, in that investigation cannabidiol was found to be inactive at concentrations of up to 100 μM, as was anandamide, whereas palmitoylethanolamide (see above) did protect from glutamate-induced neurotoxicity. More recently, Shen & Thayer[65] obtained evidence that CP55940 and (+)-WIN55212 can act through CB_1 receptors to prevent neurotoxicity by inhibiting glutamate release from hippocampal neurones. However, neither of these cannabinoid receptor agonists was found to be effective against neurotoxicity induced in these neurones by exogenously applied glutamate. Evidence also exists that Δ^9-THC can act through CB_1 receptors in hippocampal neurones to produce signs of apoptosis in these cells, possibly through the release of arachidonic acid which then acts as a source of free radicals.[66] Similarly, Sanchez *et al.*[67] have reported that Δ^9-THC can induce apoptosis in C6.9 glioma cells, albeit through a CB_1 receptor-independent mechanism.

Like most drugs, cannabinoids have adverse effects.[49,68] These include some of the changes in perception, mood, emotion and cognition that together constitute the psychotropic effects of cannabis and CB_1 receptor agonists.[68,69] More specifically, after cannabis has been taken there are reports that colours seem brighter and music more pleasant and that "felt time" passes more slowly than "clock time". Effects on mood and emotion vary. Usually there is some euphoria. However, sometimes, particularly in the inexperienced, mood may be unaffected or there may be dysphoria or anxiety. More serious adverse psychopharmacological responses can also occur, for example, panic reactions and psychoses,[68,70,71] and it would be unwise to give psychotropic cannabinoids to patients with schizophrenia (overt or latent). Signs of cannabinoid-induced changes in cognitive function include difficulty in concentrating and thinking, and impairment of memory.[69,72] Associated with the psychotropic effects of cannabinoids are reductions in psychomotor coordination and performance and changes in autonomic, endocrine and reproductive function.[68,69,73,74] The most prominent autonomic changes are cardiovascular, particularly tachycardia, postural hypotension and supine hypertension.[68,75] Consequently, patients with coronary arteriosclerosis or congestive heart failure should not take psychotropic cannabinoids.

Withdrawal of cannabis or of psychotropic cannabinoid administration can precipitate abstinence signs in man. However, these signs are both transient and mild and their significance when cannabinoids are used clinically remains to be established.[68,76–78] It is noteworthy that more marked withdrawal signs can be precipitated in cannabinoid-pre-treated animals by the CB_1 receptor antagonist, SR141716A[79–84]. The extent to which cannabinoid tolerance may present problems in the clinic has also still to be determined. Thus, although it is known that tolerance to many of the pharmacological effects of cannabinoids can be induced in animals and man,[68,76–78] the extent to which tolerance develops to the sought-after effects of cannabinoids when these are administered at therapeutic dose levels is not. The clinical significance of the ability of cannabinoids to retard fetal development and to induce fetal resorption in animals remains to be established.[68] Because of the tars produced during the combustion process, cannabis smoke may be carcinogenic and can also injure the bronchial mucosa, decrease airway conductance and impair antibacterial activity of alveolar macrophages.[68,85]

A number of strategies can be envisaged for reducing or abolishing the psychotropic effects of cannabinoids without attenuating their clinically desirable effects. One of these is to administer an agonist (partial agonist) such as 6′-cyanohex-2′-yne-Δ^8-THC[86] that has high affinity for CB_1 receptors but a reduced ability (efficacy) to activate these receptors. The underlying assumption of this approach is that therapeutic effects of cannabinoids can be achieved with a lower CB_1 receptor occupancy than is required to produce the full range of cannabimimetic psychotropic

effects. This might be because neurones mediating therapeutic effects of cannabinoids express more CB_1 receptors or contain CB_1 receptors that are more efficiently coupled to their effector systems than neurones mediating the psychotropic effects of cannabinoids. Whether this assumption is valid remains to be established. However, it is noteworthy that there are already known to be significant regional variations within the brain, both in the concentration and in the coupling efficiency of cannabinoid receptors.[2,41,87,88] Moreover, multiple sclerosis patients sometimes claim that cannabis can relieve their symptoms at dose levels that do not induce a "high". For some clinical applications it may be worth using the intrathecal route, there being evidence that intrathecal administration of CB_1 receptor agonists induces antinociception in animals.[89,90] This strategy is already sometimes adopted for baclofen, to reduce the incidence of its adverse effects in multiple sclerosis patients. If it proves that some therapeutic effects of cannabinoids are mediated by peripheral CB_1 receptors, it will also be worth designing cannabinoids that do not readily cross the blood-brain barrier but retain the ability to activate CB_1 receptors outside the central nervous system. Such effects may include analgesia, there being evidence from animal studies that cannabinoids can induce antinociception by acting on CB_1 receptors not only within the CNS[91,92] but also peripherally.[45,93] Animal experiments have also shown that cannabinoids undergo synergistic interactions with opioids for the production of antinociception[94,95] and with benzodiazepines or baclofen for inhibition of motor function.[96,97] Whether it would be of therapeutic advantage to administer a cannabinoid in combination with an opioid, a benzodiazepine or baclofen will depend on whether these agents interact synergistically with cannabinoids in man for pain or spasm/spasticity relief and on the extent to which the unwanted effects of cannabinoids, opioids, benzodiazepines and/or baclofen are augmented after combined administration. Another strategy for minimizing the unwanted central effects of cannabinoids may be to develop drugs that activate the endogenous cannabinoid system indirectly by selectively inhibiting the tissue uptake or metabolism of endocannabinoids so as to increase their levels at cannabinoid receptors. These drugs should be more selective than direct agonists as they are unlikely to affect all parts of the endogenous cannabinoid system at one time, producing instead effects only at sites where ongoing production of endogenous cannabinoids is taking place. As the enzymic hydrolysis of endocannabinoids seems to occur intracellularly, uptake inhibitors such as AM404 (see above) may have a greater impact on the concentration of endocannabinoids at their receptors than inhibitors of endocannabinoid metabolism. Finally, it may be worth searching for additional types or subtypes of CB_1 receptors as it remains possible that therapeutic effects of cannabinoids are mediated by a different CB_1 receptor subtype than their psychotropic effects.[98]

When taken orally, Δ^9-THC seems to undergo variable absorption from the gastrointestinal tract and to have a narrow "therapeutic window".[49,99] This may account for anecdotal claims that cannabis is superior to Δ^9-THC as a medicine as the comparison is usually between smoked cannabis (fast, reliable absorption) and oral Δ^9-THC (slower, less reliable absorption). It is also possible that, in addition to Δ^9-THC, there are other constituents of cannabis that contribute to its putative beneficial effects either directly or by modulating the effects of Δ^9-THC. In line with this possibility, are reports that two non-psychotropic constituents of cannabis, cannabidiol and cannabichromene, can potentiate Δ^9-THC-induced antinociception in mice.[100,101] However, there is another report that cannabidiol attenuates Δ^9-THC-induced antinociception in mice.[102] In human subjects, cannabidiol has also been found to attenuate some effects of Δ^9-THC, for example anxiety.[103,104] Clearly it will be important to resolve the question of whether Δ^9-THC is ever more effective as a medicine or has fewer or less intense unwanted effects when administered with other cannabinoids in predetermined proportions (e.g. as a preparation of cannabis) than when administered alone by the same route.

The evidence that Δ^9-THC is not be well-absorbed when taken orally prompts a need to consider the development of additional formulations and modes of administration for this agent, possibilities including administration by rectal suppository (as Δ^9-THC hemisuccinate ester),[105–107] by aerosol inhalation,[68,108–110] by skin patch or by direct application to the eye (for glaucoma) or spinal cord (see above). Quite rightly, the pressure is now on to perform clinical trials both with individual cannabinoids such as

Δ^9-THC and with cannabis. However, given the likelihood of variable absorption of Δ^9-THC from the gastrointestinal tract, there is a risk that trials performed before the development of improved formulations and modes of administration, using the oral route, may underestimate the therapeutic potential of cannabinoids by failing to detect adequate efficacy in enough patients or by indicating an occurrence of unacceptable side effects in too many patients. It would be sad, indeed, if cannabinoids were to be rejected as medicines, not because they possess serious pharmacological deficiencies, but rather because they had been tested using an inappropriate mode of administration.

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The Cannabinoid Receptor Agonist WIN 55,212-2 Reduces D_2, but Not D_1, Dopamine Receptor-Mediated Alleviation of Akinesia in the Reserpine-Treated Rat Model of Parkinson's Disease

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The effects of the synthetic cannabinoid receptor agonist WIN 55,212-2 on dopamine receptor-mediated alleviation of akinesia were evaluated in the reserpine-treated rat model of parkinsonism. The dopamine D_2 receptor agonist quinpirole (0.1 mg/kg, ip) caused a significant alleviation of the akinesia. This effect was significantly reduced by coinjection with the cannabinoid receptor agonist WIN 55,212-2 (0.1 and 0.3 mg/kg). The simultaneous administration of the cannabinoid receptor antagonist SR 141716A (3 mg/kg, ip) with quinpirole and WIN 55,212-2 blocked the effect of WIN 55,212-2 on quinpirole-induced alleviation of akinesia. The selective dopamine D_1 receptor agonist chloro-APB (SKF82958, 0.1 mg/kg) alleviated akinesia in a significant manner. WIN 55,212-2 (0.1-1 mg/kg, ip) did not affect the antiakinetic effect of chloro-APB. Combined injection of both D_1 and D_2 dopamine receptor agonists (both at either 0.1 or 0.02 mg/kg) resulted in a marked synergism of the antiakinetic effect. WIN 55,212-2 (0.1–1 mg/kg) significantly reduced the antiakinetic effect of combined injections of quinpirole and chloro-APB at both 0.1 and 0.02 mg/kg. The effect of 0.3 mg/kg WIN 55,212-2 on combined D_1 and D_2 agonist-induced locomotion (0.02 mg/kg) was blocked by SR 141761A (3 mg/kg). Neither WIN 55,212-2 alone (0.1 and 0.3 mg/kg) nor SR 141716A (3 and 30 mg/kg) alone had an antiparkinsonian effect. These results suggest that cannabinoids may modulate neurotransmission in the pathway linking the striatum indirectly to basal ganglia outputs via the lateral globus pallidus and the subthalamic nucleus. © 1997 Academic Press

INTRODUCTION

The presence of cannabinoid receptors in the basal ganglia (9, 10) has led to several investigations showing that cannabinoids modulate neurotransmission in the basal ganglia and that such actions play a key role in the control of movement (17, 18, 22; for review see 7 and 20). Cannabinoid receptors are found in especially high concentrations in both the lateral and the medial segments of the globus pallidus (GPl and GPm) and the substantia nigra pars reticulata (SNr) (10, 15). In these regions, cannabinoid receptors are localized, in part at least, on terminals of GABAergic inputs from the striatum (10). In GPl, cannabinoid receptor activation modulates GABAergic transmission by decreasing GABA reuptake from the efferent striatal terminals (17, 18). Such an effect could account for the synergism between cannabinoids and GABA seen in the lateral globus pallidus (21). It is not currently known whether cannabinoids enhance GABA transmission in GPm and SNr in a manner similar to that seen in GPl, but intranigral injections of the cannabinoid receptor agonist CP 55,940 suggest that a similar positive interaction does occur in these regions (24).

GABA transmission in the globus pallidus is enhanced in Parkinson's disease (1, 16, 19), whereas GABA transmission in the SNr and GPm (entopeduncular nucleus in the rodent) is decreased. Both these abnormalities in GABA transmission lead to enhanced activity of basal ganglia outputs from GPm/SNr to motor thalamus and other nonbasal ganglia-related motor regions. Antiparkinsonian dopaminergic therapies act in the striatum to reverse these abnormalities in GABAergic transmission. Stimulation of D_1 receptors acts predominantly on the "direct" connections between the striatum and GPm/SNr. D_2 receptors are localized preferentially, though not exclusively, on the striatal projection neurons that project to GPl (see 13 for review). Striato-GPl projections influence basal ganglia outputs indirectly by GABAergic connections between GPl and GPm/SNr (8) and GPl and subthalamic nucleus (14) which in turn sends excitatory projections to GPm/SNr (27, 4).

Recently, behavioral studies in the unilateral 6-hydroxydopamine-lesioned rat model of Parkinson's disease (2) suggested that cannabinoids can modulate D_1 dopamine receptor-mediated effects. However, given

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that cannabinoids interact with dopamine uptake in the striatum (3, 12) it is not clear whether such effects reflect an interaction in the parkinsonian or the unlesioned side of the brain. In this study, we investigated the effects of the synthetic cannabinoid WIN 55,212-2 on the D_1-, D_2-, or D_1/D_2-mediated alleviation of akinesia in the reserpine-treated rat model of parkinsonism.

MATERIALS AND METHODS

Male Sprague–Dawley rats (220–320 g, Manchester University BSU) were housed under controlled conditions: temperature (19–21°C), humidity, and light (12-h light:12-h dark regimen, 0800–2000). Food and water were available *ad libitum*. Animals were injected with reserpine (3 mg/kg, sc) under light halothane anesthesia. After 18 h, a parkinsonian state characterized by rigidity, hunched posture, and akinesia was observed. Behavioral assessment was performed between 1000 and 1300. Prior to testing, the animals were acclimated for 1 h to the room where the locomotion monitors were kept. The locomotion monitors (Activity Monitor AM 1051, Linton Instruments, UK) consisted of an array of 13 × 19 infrared light beams spaced every 2.5 cm. A count was registered when the animal broke an infrared beam. Ambulatory locomotion was assessed by measuring the number of mobile counts, i.e., an interruption of an infrared beam when the central position of the animal changed by more than two grids in any 1-s period, and was analyzed using the Amlogger software (Linton Instruments). The movement of animals was logged every 5 min, the total number of counts being the summation of all the mobile counts registered during the 5-min observation.

Injections (ip) were administered at a volume of 1 ml/kg body wt. Following injection of drug and/or vehicle the animals were placed in the locomotion monitors and after a 5-min acclimatization period their activity was recorded for 1 h. All animals were injected only once with drug/vehicle. The cannabinoid receptor agonist WIN 55,212-2 (RBI, UK) and antagonist SR 141716A (Sanofi Recherche, France) were dissolved in DMSO and then diluted with sterile water to the final concentration (maximal concentration of DMSO, 0.4%). The D_2 receptor agonist quinpirole (RBI, UK) and the D_1 receptor agonist Cl-APB hydrobromide (SKF82958, RBI, UK) were dissolved in sterile water. Statistical analysis was performed using a one-way ANOVA followed by a Tukey–Kramer post hoc test where appropriate. Significance was assumed when $P < 0.05$.

RESULTS

Effect of WIN 55,212-2 on Reserpine-Induced Akinesia

WIN 55,212-2 (0.1 and 0.3 mg/kg) did not significantly affect the locomotion in reserpine-treated animals when compared with reserpine-treated animals either injected or noninjected with the vehicle ($P > 0.05$, ANOVA, $F = 0.71$, $df = 50$) (Table 1).

TABLE 1

Effects of WIN 55,212-2 Administered Alone in Reserpine-Treated Rats

	Noninjected	Vehicle	WIN 55,212-2, 0.1 mg/kg	WIN 55,212-2, 0.3 mg/kg
Mobile counts	13 ± 4	6.9 ± 2.2	14.1 ± 9.1	5 ± 2.7
n	18	10	11	12

Note. Reserpine (3 mg/kg, sc) was administered 18 h prior to behavioral assessment. No significant difference was observed between the reserpine-treated animals that were injected with vehicle or WIN 55,212-2 and the reserpine-treated noninjected animals. The mobility of the animals is expressed as mobile counts ± SEM.

Effect of WIN 55,212-2 on Quinpirole-Induced Alleviation of Akinesia

The D_2 receptor agonist quinpirole (0.1 mg/kg) caused a significant increase in locomotion when compared with the vehicle (391 ± 54, $n = 14$, compared to 6.9 ± 2.2, $n = 10$, $P < 0.001$) (Fig. 1). Coadministration

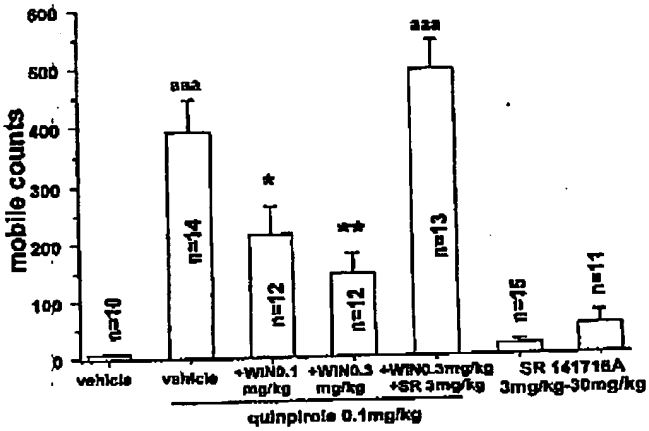

FIG. 1. Effect of the synthetic cannabinoid receptor agonist WIN 55,212-2 on D_2 receptor agonist (quinpirole)-mediated alleviation of akinesia in the reserpine-treated rat model of parkinsonism. Reserpine (3 mg/kg, sc) was administered 18 h prior to behavioral assessment. The locomotion of the animals is expressed as mobile counts ± SEM. Quinpirole (0.1 mg/kg) significantly alleviated the akinesia when compared with vehicle injection ($P < 0.001$). Coadministration with WIN 55,212-2 at doses of 0.1 and 0.3 mg/kg reduced the locomotor counts in a significant manner ($^{aaa}P < 0.01$ cf. vehicle alone; $^{*}P < 0.05$ cf. quinpirole 0.1 mg/kg; $^{**}P < 0.01$ cf. quinpirole 0.1 mg/kg). The cannabinoid receptor antagonist SR 141716A blocked the inhibitory effect obtained with 0.3 mg/kg WIN 55,212-2. Alone, SR 141716A (3 and 30 mg/kg) did not cause any increase in the locomotion counts. Statistical analysis was performed using a one-way ANOVA followed by a Tukey–Kramer multiple comparison test.

of the cannabinoid receptor agonist WIN 55,212-2 with quinpirole caused a significant decrease in quinpirole-induced locomotion ($P < 0.01$, ANOVA, $F = 31.55$, $df = 93$). This effect was greater for 0.3 mg/kg (63.1% reduction) compared with 0.1 mg/kg (45.2% reduction) (Fig. 1).

The reduction in quinpirole-induced locomotion seen with 0.3 mg/kg WIN 55,212-2 was completely blocked by coinjection with the cannabinoid receptor antagonist SR 141716A (3 mg/kg) ($P > 0.05$). SR 141716A (3 and 30 mg/kg) failed to affect locomotion (17 ± 6.6, $n = 15$ and 49 ± 22.3, $n = 11$, respectively) on its own when compared to the vehicle-treated group ($P > 0.05$, ANOVA, $F = 2.71$, $df = 35$). The antiakinetic effect of quinpirole (0.1 mg/kg) was time dependent (data not shown), resulting in a peak of locomotor activity at 20 min. This effect gradually diminished and locomotion returned to preinjection levels of locomotion at the end of the 60 min. The profile of the time course of the antiakinetic effect of quinpirole was restored and no significant difference was found between the antiakinetic effect of quinpirole (0.1 mg/kg) and of quinpirole (0.1 mg/kg) with WIN 55,212-2 (0.3 mg/kg) and SR 141716A (3 mg/kg) ($P > .05$, Student t test).

Effect of WIN 55,212 on Cl-APB-Induced Alleviation of Akinesia

The D_1 agonist Cl-APB hydrobromide (0.1 mg/kg) caused a significant alleviation of the akinesia (259 ± 54, $n = 15$, $P < 0.05$) compared with the vehicle. Unlike quinpirole, this effect was not altered by coinjection with WIN 55,212-2 (0.1–1 mg/kg) (Fig. 2). No other obvious behavioral effect was observed in the presence of the cannabinoid receptor agonist WIN 55,212-2 at doses from 0.1 to 1 mg/kg.

Effect of WIN 55,212-2 on D_1- and D_2-Mediated Alleviation of Parkinsonism

Combined injection of quinpirole (0.1 mg/kg) and Cl-APB hydrobromide (0.1 mg/kg) resulted in a marked potentiation of the antiakinetic effect (8529 ± 866, $n = 18$) compared to either alone (259 ± 54, $n = 15$, $P < 0.001$ for Cl-APB and 391 ± 54, $n = 14$, $P < 0.001$ for quinpirole). This stimulation of locomotion lasted for over 1 h and was characterized by pronounced hyperactive stereotyped behavior. WIN 55,212-2 (0.3 and 1 mg/kg) reduced the hyperactive locomotion and behavior in a significant manner. WIN 55,212-2 (0.3 mg/kg) reduced the mobile counts by 45.8% whereas WIN 55,212-2 (1 mg/kg) reduced mobile counts by 83.2% (Fig. 3a). Combined injections of quinpirole (0.02 mg/kg) and Cl-APB hydrobromide (0.02 mg/kg) resulted in a significant alleviation of akinesia (1155 ± 343, $n = 15$). WIN 55,212-2 (0.1 and 0.3 mg/kg) decreased the antiakinetic effect of quinpirole and Cl-APB hydrobromide in a significant manner ($P < 0.001$, ANOVA, $F = 49.6$, $df = 120$). The effect of 0.3 mg/kg WIN 55,212-2 on the quinpirole and Cl-APB hydrobromide alleviation of the akinesia ($P < 0.001$, 91% reduction) was blocked by coadministration of SR 141716A (3 mg/kg) ($P > 0.05$) (Fig. 3b).

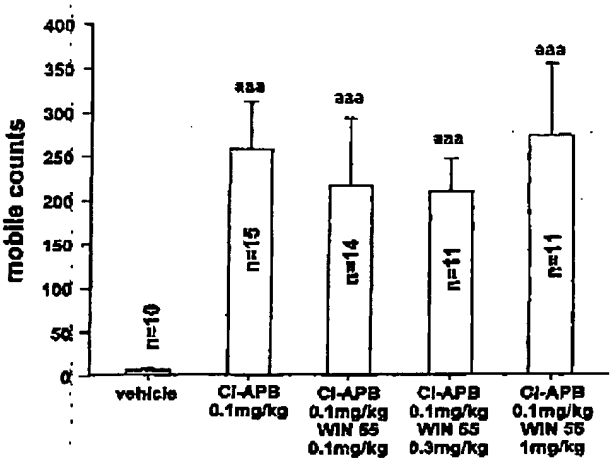

FIG. 2. Effect of the synthetic cannabinoid WIN 55,212-2 on the D_1 receptor agonist (Cl-APB hydrobromide)-induced alleviation of akinesia in the reserpine-treated rat model of parkinsonism. Reserpine (3 mg/kg, sc) was administered 18 h prior to behavioral assessment. The locomotion of the animals is expressed as mobile counts ± SEM. Cl-APB (0.1 mg/kg) significantly alleviated the akinesia when compared to the vehicle group (***$P < 0.001$, ANOVA, Tukey–Kramer). However, coinjection with WIN 55,212-2 (0.1–1 mg/kg) did not affect Cl-APB-induced locomotion ($P > 0.05$ cf. Cl-APB alone).

DISCUSSION

This study demonstrates that cannabinoid receptor activation decreases dopamine receptor-induced alleviation of akinesia in the reserpine-treated rat model of Parkinson's disease. This effect is specific for D_2 receptor-mediated antiparkinsonian effects.

In this study, the reserpine-treated rat model of Parkinson's disease was used to evaluate the effect of cannabinoids on the antiparkinsonian effects of dopamine receptor stimulation. This model was preferred to the unilateral 6-OHDA-lesioned rat to avoid misinterpretation of results due to effects of cannabinoids on the unlesioned side of an animal model such as the 6-OHDA-lesioned rat.

In the 6-OHDA-lesioned rat, rotational behavior contraversive to the lesion reflects net overactivity of the output regions of the basal ganglia (entopeduncular nucleus/substantia nigra pars reticulata, i.e., EP/SNr) on the lesioned compared to the unlesioned side. Thus, the contraversive circling seen following dopamine receptor activation which is normally taken as indica-

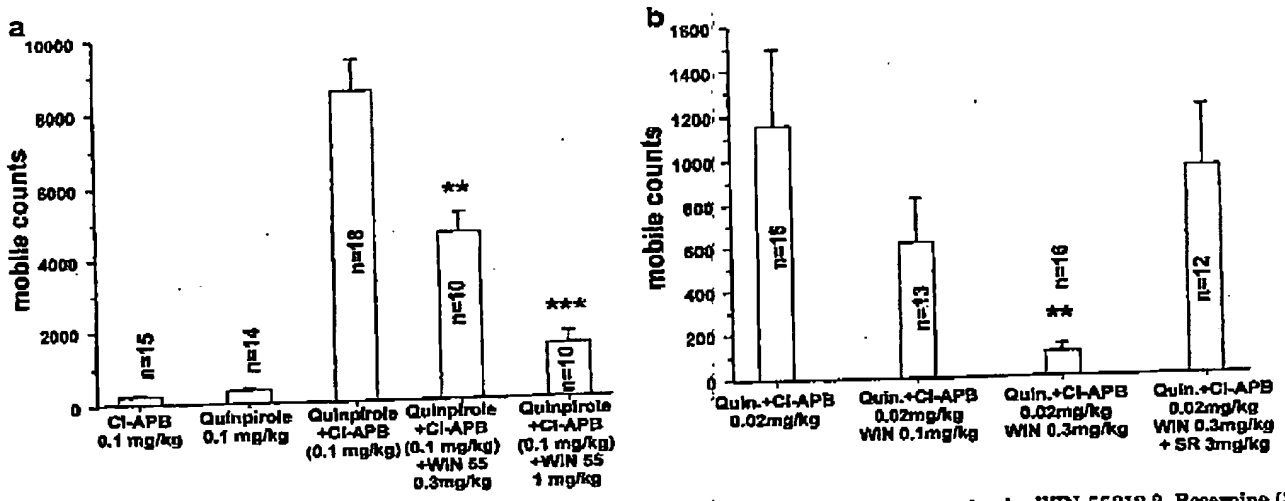

FIG. 3. Synergistic effect of quinpirole (0.1 mg/kg) and Cl-APB (0.1 mg/kg) on locomotion: attenuation by WIN 55212-2. Reserpine (3 mg/kg, sc) was administered 18 h prior to behavioral assessment. The locomotion of the animals is expressed as mobile counts ± SEM. (a) A very marked increase in locomotion was observed following combined injection of quinpirole and Cl-APB at doses that had significant antiparkinsonian actions when given alone (see Figs. 1 and 2). WIN 55,212-2 (0.3 and 1 mg/kg) reduced this locomotion in a significant manner (**$P < 0.01$ cf. quinpirole and Cl-APB; ***$P < 0.001$ cf. quinpirole and Cl-APB). (b) Combined injection of a lower, subthreshold, dose of quinpirole and Cl-APB (both 0.02 mg/kg) significantly alleviated the akinesia when compared with the vehicle-tretated group ($P < 0.001$) while reducing the hyperactive behavior in a significant manner (**$P < 0.011$. The effect of 0.3 mg/kg WIN 55,212-2 was abolished by coadministration with SR 141716A (3 mg/kg). No significant difference was found between quinpirole + Cl-APB (0.02 mg/kg) and quinpirole + Cl-APB (0.02 mg/kg) + WIN 55,212-2 (0.3 mg/kg) + SR 141716A (3 mg/kg) ($P > 0.05$).

tive of an antiparkinsonian effect results from a decrease in the firing rate of EP/SNr neurons on the lesioned side. However, similar behavior could be seen if the activity of the EP/SNr on the unlesioned side was increased. Given the fact that cannabinoids can modulate dopamine uptake (11, 23) and would, in this respect, act preferentially on the unlesioned side, it makes the interpretation of reports of cannabinoids reducing dopamine D_1 receptor agonist-induced rotation difficult (2).

WIN 55,212-2 had no effect on locomotion when given alone. The absence of effect of WIN 55,212-2 alone on reserpine-induced akinesia was not interpreted as an additional effect of the cannabinoid on catalepsy as, at the doses employed (0.1–1 mg/kg), no catalepsy is seen in the normal rat (2). We have found that the cataleptogenic effect of WIN 55,212-2 only becomes additive with reserpine (3 mg/kg) for doses of WIN 55,212-2 greater than 5 mg/kg (data not shown). The variability of the locomotor scores observed following injection of 0.1 mg/kg WIN 55,212-2 alone was greater than for higher doses or of vehicle. The reason for this is unclear.

The lack of effect of WIN 55,212-2 (0.1–1 mg/kg) on the full D_1 agonist Cl-APB-induced alleviation of akinesia is in contrast with previous findings showing that cannabinoids decrease the D_1-mediated contralateral circling in the 6-OHDA-lesioned rat (2). The fact that it was not seen in the present study may suggest a D_1–cannabinoid interaction on the unlesioned side of the brain that would enhance basal ganglia outputs on the unlesioned side. Thus the authors' speculation that cannabinoids may decrease the D_1-stimulated GABA release from the striatal terminals in the substantia nigra nigra by acting on N-type calcium channels seems unlikely. Indeed, to date, there is no evidence of such negative modulation of GABAergic transmission. On the contrary, there is much evidence to suggest that cannabinoids enhance GABAergic transmission in the globus pallidus and SNr (17, 18, 21, 24).

WIN 55,212-2 decreased the D_2 receptor agonist quinpirole-induced alleviation of akinesia in a significant manner. This effect was blocked by coadministration with the cannabinoid receptor antagonist SR 141716A. Again this result contrasts with previous reports in unilateral models of Parkinson's disease where the cannabinoid receptor agonist CP 55,940 had no effect on D_2 receptor agonist-mediated contralateral rotation in the 6-OHDA-lesioned rat (2). The effects of D_2 stimulation on Parkinson's disease symptoms are mediated through the striatal outputs that influence basal ganglia outputs indirectly. Thus D_2 agonists reduce activity in the overactive striatopallidal pathway to GPl. This in turn leads to increased inhibition of the STN. Decreased activity of STN leads to decreased excitation of GPm/SNr which we have previously shown to reverse parkinsonian symptoms. Several potential

sites of D_2–cannabinoids interactions are thus apparent. It is unlikely that this interaction occurs in the striatum because in the unilaterally 6-OHDA-lesioned mouse, contralateral rotation induced by intrastriatal injections of cannabinoids is decreased by either D_1 or D_2 receptors (26). Actions of cannabinoids in the STN are unknown, but preliminary reports suggest that cannabinoids might reduce glutamate release from subthalamic efferents in GPm/SNr (25). The most likely site of interaction is the GPI. It has been shown that cannabinoid receptor activation can decrease GABA uptake in GPI. Such an action would tend to act to counteract the decreased GABA release induced by D_2 inhibition of the indirect striatal output pathway.

The marked synergistic effect of combined injection of D1 and D2 receptor stimulation on locomotion has been reported before using the partial D1 agonist SKF38393 (13). However, in the present study, it appears that the full D_1 agonist Cl-APB enhances the effects of D_2 stimulation to an even greater extent. WIN 55,212-2 reduced the effect on locomotion elicited by combined injection of D_1 and D_2 receptor agonists (both at sub- and at suprathreshold doses). SR 141716A blocked the inhibition of the antiparkinsonian effect. These effects probably result from an action on the D_2 stimulation as WIN 55,212-2 did not reduce locomotion to levels below that seen with the D_1 agonist alone.

A recent study showed that in mice, SR 141716A increased locomotion at doses of 10 and 30 mg/kg (5). The authors concluded to the possible existence of an endogenous cannabinoid tone that could be responsible for inhibiting movement. In our study the absence of antiakinetic effect of SR 141716A (3 and 30 mg/kg) does not seem to corroborate their results. However, as the authors point out, the high doses necessary to produce a locomotor effect could account for a novel or even nonspecific action of the cannabinoid receptor antagonist.

The present study therefore suggests an inhibitory effect of cannabinoids on D_2-mediated alleviation of akinesia in the reserpine-treated rat model of parkinsonism. We suggest that such an action takes place in the globus pallidus although further investigation is needed to fully characterize the site of action of cannabinoids. While these data suggest that cannabinoids might not prove useful in the treatment of Parkinson's disease, they do point toward opportunities for employing cannabinoids in the treatment of movement disorders.

Actions of cannabinoids that enhance GABA transmission in GPI may prove useful in the very early stages of Huntington's disease when there is degeneration of the GABAergic inputs to GPI from the striatum. In this instance, decreased GABAergic tone in the GPI manifests itself as chorea. Similar choreic symptoms are also seen in dyskinesias seen in parkinsonian patients following long-term dopamine agonist therapies. These symptoms may result in part from D_2-mediated reductions in GABA release in GPI (6). The data presented in this study suggest that cannabinoids might act to reduce D_2-mediated reductions in GABA release in GPI. Such an action would be a useful adjunct to dopamine agonists in the treatment of Parkinson's disease and may allow the antiparkinsonian effects of dopamine replacing agents without eliciting dyskinesia.

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